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DIURNAL PATTERNS OF GROWTH AND TRANSIENT RESERVES OF SINK AND SOURCE TISSUES ARE AFFECTED BY COLD NIGHTS IN BARLEY

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Abstract:	Barley is described to mostly use sucrose for night carbon requirements. To understand how the transient carbon is accumulated and utilized in response to cold, barley plants were grown in a combination of cold days and/or nights. Both daytime and night cold reduced growth. Sucrose was the main carbohydrate supplying growth at night, representing 50-60% of the carbon consumed. Under warm days and nights, starch was the second contributor with 26% and malate the third with 15%. Under cold nights, the contribution of starch was severely reduced, due to an inhibition of its synthesis, including under warm days, and malate was the second contributor to C requirements with 24-28% of the total amount of carbon consumed. We propose that malate plays a critical role as an alternative carbon source to sucrose and starch in barley. Hexoses, malate, and sucrose mobilisation and starch accumulation were affected in barley elf3 clock mutants, suggesting a clock regulation of their metabolism, however without affecting growth and photosynthesis. Altogether, our data suggests that the mobilisation of sucrose and malate and/or barley growth machinery are sensitive to cold.

DIURNAL PATTERNS OF GROWTH AND TRANSIENT RESERVES OF SINK AND SOURCE TISSUES ARE AFFECTED BY COLD NIGHTS IN BARLEY

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ABSTRACT

Barley is described to mostly use sucrose for night carbon requirements. To understand how the transient carbon is accumulated and utilized in response to cold, barley plants were grown in a combination of cold days and/or nights. Both daytime and night cold reduced growth. Sucrose was the main carbohydrate supplying growth at night, representing 50-60% of the carbon consumed. Under warm days and nights, starch was the second contributor with 26% and malate the third with 15%. Under cold nights, the contribution of starch was severely reduced, due to an inhibition of its synthesis, including under warm days, and malate was the second contributor to C requirements with 24-28% of the total amount of carbon consumed. We propose that malate plays a critical role as an alternative carbon source to sucrose and starch in barley. Hexoses, malate, and sucrose mobilisation and starch accumulation were affected in barley *elf3* clock mutants, suggesting a clock regulation of their metabolism, however without affecting growth and photosynthesis. Altogether, our data suggests that the mobilisation of sucrose and malate and/or barley growth machinery are sensitive to cold.

48

49 1 INTRODUCTION

50 Plants are growing during both day and night (Walter, Silk & Schurr, 2009), but can
51 reduce CO_2 to produce carbohydrates only in the light during photosynthesis. It is yet not clear
52 how plants can orchestrate these two major fundamental processes. Growth and photosynthesis
53 are partly temporally distinct and they are also spatially separated between source and sink
54 tissues (Ludewig & Sonnewald, 2016, Schnyder, 1993, Wang & Tillberg, 1996). Thus, to fully
55 understand the cross-talk between photosynthesis and growth, it is necessary to analyse sink
56 and source tissues separately, and also gather temporal information. The storage of
57 photoassimilates is spatially separated in source and sink tissues, not only for daily/night
58 requirements for growth and maintenance, but also over long time for e.g. supplying flowering
59 and grain development (Schnyder, 1993, Smouter & Simpson, 1991). Changes in carbohydrate
60 metabolism are vital to overcome abiotic stresses (Pommerrenig, Ludewig, Cvetkovic,
61 Trentmann, Klemens *et al.*, 2018) and as such the partitioning of photoassimilates is affected
62 by environmental factors but also developmental processes. The most studied and common
63 transient carbon (C) storage in plants is starch. However, barley, wheat and some grasses, might
64 not primarily use starch like *Arabidopsis*, *Brachypodium* or maize as a transient C store for
65 night usage, but can also use sucrose and possibly fructans (Farrar & Farrar, 1985, Nagaraj,
66 Altenbach, Galati, Luscher, Meyer *et al.*, 2004, Nagaraj, Riedl, Boller, Wiemken & Meyer,
67 2001).

68 *Arabidopsis* C reserves and diurnal growth are highly controlled by circadian clock
69 genes (Graf, Schlereth, Stitt & Smith, 2010). However, in *Zea mays* and *Oriza sativa,* growth
70 is stable over the diurnal cycle and strongly affected by temperature regimes, in contrast with
71 dicotyledonous species (Poire, Wiese-Klinkenberg, Parent, Mielewczik, Schurr *et al.*, 2010).
72 Thus, monocots and dicots might have different sensitivities regarding the respective

influences of the clock and the environment on the growth patterns, and thus likely C reserves utilisation. In other words, clock genes are conserved amongst dicotyledons and monocotyledons; however, they might not to have the same importance in the control of diurnal growth and transient C reserves amongst these groups (Müller, von Korff & Davis, 2014).

Previous studies on photoassimilates in barley showed that the main one is sucrose, with low amounts of starch and fructans (Gordon, Ryle, Mitchell & Powell, 1982, Gordon, Ryle & Powell, 1977, Gordon, Ryle & Powell, 1979, Gordon, Ryle, Powell & Mitchell, 1980a). However, the analysis of starch in barley and more generally fructan-accumulating plants has been largely neglected, so it is not yet clear how they compete for photosynthates. Moreover, no large quantitative metabolite studies over a diurnal time course have been performed, so some other important metabolites cannot be excluded. Fructans are known to play an important role on cold tolerance in fructan accumulating species (Abeynayake, Etzerodt, Jonaviciene, Byrne, Asp *et al.*, 2015, del Viso, Puebla, Fusari, Casabuono, Couto *et al.*, 2009, Jeong & Housley, 1990, Meguro-Maoka & Yoshida, 2015, Rao, Andersen, Dionisio & Boelt, 2011, Tamura, Sanada, Tase & Yoshida, 2014, Tarkowski & Van den Ende, 2015). However, little is known about the diurnal regulation of fructan levels and their potential role as transient storage of C for night usage (Schnyder, 1993). In barley, fructan mobilization at night has been suggested (Farrar & Farrar, 1985) and the accumulation at the base of young leaves has been hypothesised to supply growth of new leaves (Roth, Luscher, Sprenger, Boller & Wiemken, 1997). Sucrose has been described previously as a transient carbon store in grasses and it is also the substrate for fructan synthesis (Nagaraj *et al.*, 2004, Nagaraj *et al.*, 2001, Ritsema, Brodmann, Diks, Bos, Nagaraj *et al.*, 2009). Even though fructan synthesis is correlated to increase in sucrose levels (Nagaraj *et al.*, 2001), in another study (Jin, Fei, Rosenquist, Jin, Gohil *et al.*, 2017), the authors described a mechanism linking fructan and starch synthesis through a single gene that encodes two transcription factors named SUSIBA (sugar signalling

in barley). These transcription factors have different lengths and respond to different sucrose concentrations, acting in an antagonistic and auto-regulatory way, which result in the control of the rates of starch and fructan synthesis in barley.

A common strategy of plants to acclimate to cold is the accumulation of water soluble carbohydrates, the type of sugars accumulated varying between species (Ruelland, Vaultier, Zachowski & Hurry, 2009). The recommended sowing period for spring barley in Ireland is from late February to March. Thus, spring barley faces at a very early stage low temperature during the day and night in early spring that later changes to warmer temperatures and longer daylength through late spring and summer. Sowing date can be a determinant of the final yield of cereal crops (Conry, 1995, Conry, 1998, Potterton & McCabe, 2018). If they undergo higher temperature at tillering stage, they transition faster between developmental stages (Kirby, Appleyard & Fellowes, 1982) and may produce smaller leaves and less tillers which could impact the number of ears and consequently lower yield. Studies on sowing date for spring barley show that the earlier the sowing, the higher the yield (Conry, 1995, Conry, 1998, Kirby *et al.*, 1982, Kumar, Singh, Hooda, Sewhag & Chaudhary, 2017, Photiades & Hadjichristodoulou, 1984, Potterton & McCabe, 2018). Thus, although spring varieties are less exposed than winter varieties to cold, they still experience cold at early stage, which seems to be crucial in regulating their development (Kirby, Appleyard & Fellowes, 1985) and thus a full understanding of the response of spring barley to cold could help increase growth rates and subsequently yields. Cold and freezing tolerance in plants are achieved by a combination of increased protein content, sugars and other soluble metabolites such as compatible solutes (*e.g.* proline, betaines, sugar alcohols) or flavonoids (Al-Hamdani & Thomas, 2001, Bourion, Lejeune-Henaut, Munier-Jolain & Salon, 2003, Hurry & Huner, 1992, Janmohammadi, Mock & Matros, 2014, Lorenzo, Assuero & Tognetti, 2015, Oquist, Hurry & Huner, 1993, Savitch, Harney & Huner, 2000, Trischuk, Schilling, Low, Gray & Gusta, 2014, Tyrka, Rapacz, Fiust,

Wójcik-Jagła & Rognli, 2015, Visioni, Tondelli, Francia, Pswarayi, Malosetti *et al.*, 2013). Freezing and cold tolerance are mainly orchestrated by C-REPEAT-BINDING FACTOR (CBF) genes (Cook, Fowler, Fiehn & Thomashow, 2004, Pare, Gilmour, Grumet & Thomashow, 2018, Shi, Ding & Yang, 2018, Thomashow, 1999, Thomashow, 2010). The circadian clock also seems to be part of the pathway regulating cold acclimation in Arabidopsis, with a number of metabolites involved in cold acclimation showing circadian oscillations under free running cycles in the cold, and clock mutants exhibiting impaired freezing tolerance (Espinoza, Degenkolbe, Caldana, Zuther, Leisse *et al.*, 2010). Among clock mutants, *EARLY FLOWERING 3* (*elf3*) has been involved in growth and temperature responses (Box, Huang, Domijan, Jaeger, Khattak *et al.*, 2015, Ford, Deng, Clausen, Oliver, Boden *et al.*, 2016).

To obtain a better understanding of the temporal and spatial mobilization of transient C stores to supply growth at night when spring varieties are still at early developmental stages, and analyse the effects of cold treatments, we grew barley seedlings in three thermo regimes, warm days and nights (22ºC:18ºC), warm day and cold nights (22ºC:4ºC) and cold days and nights (10ºC:4ºC). We characterised photosynthetic traits and growth of the plants. Sink and source tissues above ground were harvested during a 24 h time course and analysed for their content in primary metabolites. Because the involvement in cold tolerance by the clock has been suggested, we also included in our study *elf3* spring barley mutants.

2 MATERIAL AND METHODS

2.1 Plant material, growth conditions and harvest

Barley seeds (*Hordeum vulgare* L.) of spring variety Propino were germinated in darkness at 24 ºC for 3 days on dampened paper. One seedling was transferred per pot, filled with Bord na Móna potting substrate plus^{+} (Bord na Móna Horticulture Ltd., Ireland, and all pots were transferred to a growth chamber (LED-36HVL LT, Percival Scientific, Inc., USA).

Plants were submitted to three temperature conditions: warm day and night at 22 ºC:18 ºC as control, cold day and night at 10 ºC:4 ºC and cold only at night at 22ºC:4ºC; under 500 µmol photons and a photoperiod of 12h:12h light:dark for all conditions. Plants were harvested when they reached 3 leaf stage, with the third leaf – youngest leaf – being 3 to 5 cm above the ligula. The middle section of sheaths and blades of each leaf were harvested separately. Three replicates were harvested at five timepoints covering a period of 24 h, each replicate consisting of the pooled sheaths or blades from three different plants. Samples were frozen in liquid nitrogen, grinded to fine powder and then stored at -80 ºC for metabolic analyses

Seeds of the spring barley cv. Bowman and introgression lines 289 and 290 in this cultivar, that carries introgression of the *eam8.k* allele, were germinated in dark at 24 ºC for 3 days on dampened paper and then transferred to growth chamber equipped with LED lights (C75-NS1, C75-AP67, Valoya, Finland) into pots with Bord na Móna potting substrate plus^{+} (Bord na Móna Horticulture Ltd., Ireland). The *eam8.k* allele is characterized by a base-pair mutation leading to a premature stop codon in *HvELF3*, which is orthologous to *ELF3* in Arabidopsis (Faure, Turner, Gruszka, Christodoulou, Davis *et al.*, 2012). Each introgression line was grown with WT at 500µmol photons m^{-2}, 22 ºC:18 ºC, 10 ºC:4 ºC and 22ºC:4ºC day:night; and a photoperiod of 12 h:12 h light:dark. Genotypes were randomly distributed in the chamber and three replicates were harvested at five timepoints covering a period of 24 h, each replicate consisting of 3 pooled sheaths or blades from different plants with third leaf – youngest leaf – being 3-5 cm above the ligula. Samples were freeze-dried, grinded to fine powder and then stored in container with silica gel.

Crowns of five plants were harvested at end of light and dark periods of all temperature combinations for Propino, Bowman WT and introgression lines, frozen in liquid nitrogen, grinded to fine powder and then stored at -80 ºC for metabolic analyses.

2.2 Elongation rate and chlorophyll fluorescence parameters

Second and third blades were marked at the base of the blade at 0 h, then at end of night period (12 h) were marked again at the base of the blade and lastly at end of day (24 h). The elongation rate was calculated by the difference of each period's measurement divided by the duration in hours of the period: 12 h for night and 12 h for day.

2.3 Chlorophyll fluorescence parameters and gas exchange

Chlorophyll fluorescence parameters were taken using a PAM-2500 (Heinz Walz GmbH, Germany). The maximum photochemical quantum yield of PSII (F_v/F_m) and the effective photochemical quantum yield of PSII (Y(II)) were determined at steady state of chlorophyll fluorescence with a saturation pulse of 8.000 µmol $m^{-2}s^{-1}$ (Genty, Briantais & Baker, 1989, Kitajima & Butler, 1975). ETR was calculated according to PAM-2500 handbook guidelines.

The net photosynthesis (A_N), the stomatal conductance (g_s), sub-stomatal CO_2 concentrations and transpiration (E) were measured in open system infra-red gas exchange (LI-6400XT, LI-COR, Lincoln, NE, EUA). The temperature of the chamber was kept at 22 °C for warm day plants and 10 °C for plants under cold day, the gas chamber being temperature controlled. The vapour pressure deficit (VPD) was kept around 1.1 kPa, the CO_2 concentration was set at 400 ppm, light flux set to 500 µmol photons.m^{-2}.s^{-1} of photosynthetic active radiation (PAR). Measurements were taken on the second blade for all temperature conditions.

2.4 Metabolites determination

For metabolic analyses, 20 mg of frozen powder was submitted to ethanolic extraction. Sequential extractions with ethanol concentrations of 98%, 80% and 50% were performed and between each step the samples were incubated at 85ºC for 20 min and centrifuged at 3220 *g*

196 for 10 min . The ethanolic phase was used to determinate soluble sugars and malate while starch
197 and proteins were determined in the pellet. Glucose and fructose were determined according to
198 with minor modifications. We used 0.6 $U.\mu l^{-1}$ NAD^+ dependant G6PDH and the determination
199 of sucrose was performed using 0.25 $U.\mu l^{-1}$ α-glucosidase (E-MALTS, Megazyme u. c.,
200 Ireland). The production of NADH was determined at 340 nm using a spectrophotometer model
201 ELx800™ (BioTek Instruments, Inc., USA).

202 Fructans were determined after completion of sugar analyses, using the same
203 determination plate. The NADH and enzymes used for sugar analyses present in the wells were
204 hydrolysed by addition of 10 µl HCl 1 M and the plate was sealed and incubated at 95 ºC for
205 30 min. Then the plate was cooled on ice and extracts neutralized with 10 µl NaOH 1 M. To
206 each well, 7 µl of acetate buffer 0.1 M pH 4.9 were added to the plate and 1 µl of a mix
207 containing 0.1 $U.\mu l^{-1}$. endo-inulinase and 0.1 $U.\mu l^{-1}$ exo-inulinase (respectively E-ENDOIAN,
208 E-EXOIAN, Megazyme u. c., Ireland). The plate was then sealed and incubated overnight at
209 37 ºC. To determine fructans, 75 µl of Hepes buffer 0.5 M pH 7 containing 3 mM ATP and 1.3
210 mM NAD was added in each well. After obtention of a sTable baseline at 340 nm, 1 µl of 0.6
211 $U.\mu l^{-1}$ glucose-6-phosphate dehydrogenase, 1 µl 0.9 $U.\mu l^{-1}$ hexokinase and 1 µl 0.3 $U.\mu l^{-1}$
212 phosphoglucose isomerase were added sequentially for the determination of glucose and
213 fructose molecules present in fructans. Starch was determined as previously described by
214 Hendriks, Kolbe, Gibon, Stitt and Geigenberger (2003). Malate was determined according to
215 Cross et al. (2006). Proteins were determined by the method described by Lawry et al. (1951),
216 adapted to 96-well plate.

217 **2.5 Water content and carbon content estimations**

218 The water content was determined on the second blade. Five leaf discs per blade were
219 collected on six plants. The 30 discs were excised and immediately weighed. Then the discs

were dried in a drying cabinet at 70 ºC for 72 h and weighed again. Then the difference was used to calculate the percentage of water and dry matter per gram of fresh weight.

For the calculation of carbon accumulation and consumption, we used the metabolite content determined at end of day and end of night in the different plant organs, multiplying the concentration of metabolite by the number of carbon atoms present in each molecule, i.e. 6 for glucose, fructose, sucrose (equivalent glucose), fructans (equivalent glucose), starch (equivalent glucose) and 4 for malate. Then, C concentration at end of day (μmol $C.g^{-1}$ FW) and C consumption at night (μmol $C.g^{-1}$ FW) were estimated at whole plant levels by taking into account the respective weights of each organ per plant. The carbon consumption at night was estimated by the difference between content found at the first, last (end of night, EN) and third time point (end of day, ED).

2.6 Statistical analysis

For the comparisons between ED and EN for elongation rates and metabolite levels, independent t-test were carried out, using six replicates. For comparison of temperature treatments and genotypes, , ANOVA was applied followed by Tukey test, using 3-6 replicates. All tests were conducted on IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, IBM Corp. Means were considered significantly different at $P<0.05$.

3 RESULTS

3.1 Growth is reduced by cold, proteins are only affected by daytime cold, and chlorophyll fluorescence parameters are only marginally affected

Barley grown under 10 ºC:4 ºC temperature regime presented a different physiological response compared to plants grown under 22 ºC:18 ºC and 22 ºC:4 ºC temperature regimes, with a significant decrease in their height (Figure S1a) despite a similar biomass (Figure S1b). Moreover, plants grown under 10 ºC:4 ºC showed a lower leaf 2 water content (Table 1)

compared to other conditions. For all conditions the fluorescence parameters were similar (Table 1), at the exception of a significant small decrease in F_v/F_m, Y(II) and ETR observed in plants grown under cold day and night. Highest protein contents were observed in blades of all leaves and the sheath of the third leaf for all treatments (Figure 1). Protein levels did not show any diurnal turnover. In warm daytime and cold night, the protein levels were similar to those observed under warm daytime and night (Figure 1a-b). However, when plants were grown in cold daytime and night, protein levels increased in all tissues (Figure 1c). All plants were harvested at the same stage of development, with the third leaf being exposed by 3cm at least and not more than 5 cm. Plants grown in 22 ºC:18 ºC reached this stage 15 days after sowing (DAS), while plants under 10 ºC:4 ºC took 42 DAS, and 22 ºC:4 ºC plants 20 DAS. Elongation rates at night were lower than during daytime for all treatments. Second leaves presented lower elongation rate (Figure 2a) than third leaves (Figure 2b) in all conditions, suggesting that second leaves were reaching maturity. Plants grown under 10 ºC:4 ºC showed on average a 75% reduction in the elongation rates of their second and third leaves compared to control condition (Figure 2). Plants submitted to cold only at night showed similar elongation rates to the 22 ºC:18 ºC treatment during the daytime, but at night elongation rates were comparable to plants grown under 10ºC:4ºC.

3.2 Diurnal patterns of soluble sugars, fructans, starch and malate are affected by temperature

Blades contained very low concentrations of both glucose and fructose, but sheaths contained higher levels. Glucose and fructose predominantly accumulated in the youngest sheath (leaf 3) for the three temperature regimes. However, glucose and fructose were only almost fully consumed under the 22 ºC:18 ºC treatment (Figure S2a and Figure S3a, all metabolic data in Table S1) and their turnover was strongly inhibited by the cold night plants faced in the 10 ºC:4 ºC and 22 ºC:4 ºC temperature regimes (Figure S2b-c and Figure S3b-c).

When cold was present during day and night, glucose and fructose turnover was totally abolished in leaves while a small turnover was only observed for glucose when plants were grown under the 22 ºC:4 ºC temperature regime.

Sucrose content in sheaths of plants grown in the three conditions was low and almost no turnover was observed (Figure 3). In contrast, sucrose predominantly accumulated in old blades, and a high turnover was observed for all three temperature regimes. Under the 22 ºC:18 ºC temperature regime, higher sucrose and starch levels were observed at ED2 compared to ED1, in particular for the youngest leaf, likely due to the blades getting mature. Temperature had an effect on sucrose accumulation, with the blades of plants grown under the 10 ºC:4 ºC temperature regime exhibiting almost twice the concentration of sucrose observed in the blades of the plants grown under 22 ºC:18 ºC (Figure 3a and 3c). Interestingly, when the cold was applied only at night (22ºC:4ºC), the accumulation and turnover of sucrose in the blades was very similar to those of plants grown under the 22 ºC:18 ºC temperature regime (Figure 3b).

Fructan levels were not affected in the same manner as sucrose (Figure S4). Plants grown under warm day and night conditions and those which experienced only cold nights accumulated very low amounts of fructans in both blades and sheaths, and no turnover was observed (Figure S4a-b). A small accumulation of fructans was observed in the oldest blade and youngest sheath of plants grown under the 10 ºC:4 ºC treatment (Figure S4c). It is different from sucrose that accumulated predominantly in older blades, but not in sheaths. Despite the low levels of fructans accumulated in first blade and third sheath, the turnover of fructans observed in these tissues was about 50%.

Plants grown under warm days and nights showed the highest starch accumulation, mainly in the second and third blades (Figure 4a) and starch was nearly exhausted by the end of the night. In the presence of cold treatment, either both in daytime and night or solely at night, starch accumulation was reduced to less than 25% of the levels observed in blades of

294 plants grown under 22 ºC:18 ºC (Figure 4b-c). However, starch was still almost fully consumed
295 at night for both cold treatments.

296 Malate showed different patterns according to the temperature in which plants were
297 grown. Plants under 22 ºC:18 ºC showed increased malate concentration in the third sheath and
298 blade as well as a small turnover (Figure 5a), with a small accumulation in other tissues. Under
299 warm daytime and cold nights, plants showed an intermediary accumulation of malate in the
300 first and second leaves compared to 22 ºC:18 ºC and 10ºC:4ºC, and highest levels in both blades
301 and sheaths of the third leaves, so in the youngest tissues (Figure 5b). When plants were
302 submitted to 10 ºC:4 ºC, high levels of malate were again observed in youngest tissues, but also
303 in the blade of leaf 2, malate being consumed in blades at night (Figure 5c).

304 **3.3 Metabolite accumulation in the daytime and their consumption at night are**
305 **modified in source tissues to supply growth in cold nights**

306 Plants grown under 22 ºC:4 ºC accumulated the highest amounts of metabolites at the
307 end of the day while plants under 10 ºC:4 and 22 ºC:18 ºC ºC accumulated similar levels (Table
308 4). Sucrose followed by starch and malate were the main metabolites accumulated during the
309 light period for warm day and nights, but malate was second major metabolite for plants grown
310 under warm days and cold nights and cold days and nights. Starch accumulation was drastically
311 reduced in plants grown under the 10 ºC:4 ºC (67%) and 22 ºC:4 ºC (43%) temperature regimes
312 compared to 22ºC:18ºC, and this decrease was compensated by an increase in malate content
313 compared to 22 ºC:18 ºC (Table 4).

314 The consumption of C at night by 22 ºC:18 ºC control plants was around 94% of the
315 total C accumulated during the day, while for plants grown under 10 ºC:4 ºC the consumption
316 was 74% and significantly reduced for plants grown under 22 ºC:4 ºC with 64% (Table 4). The
317 proportion of each metabolite consumed under the three temperature regimes was very similar
318 to the proportion of metabolites accumulated at the end of the day, with sucrose being the main

contributor. Starch was the second contributor under warm days and nights, but malate was more used at night under the two cold treatments, being particularly important under 22 ºC:4 ºC, contributing with 28% of the total carbon consumed, despite a stark decrease in the turnover of malate (Table 4). Indeed 84% of the malate accumulated at ED was consumed during 22 ºC:18 ºC, but only 53% during cold nights in the 22ºC:4ºC temperature regime.

In general, sheaths had a low contribution to the supply of carbon for night use, at the exception of the sheaths of third leaf where malate and glucose were the main providers of carbon (Figure 6). Under 22 ºC:18 ºC, the oldest blade provided predominantly sucrose, while the second blade provided almost equally sucrose and starch and the third blade sucrose, starch and malate (Figure 6a). However, when plants are submitted to 10 ºC:4 ºC, all blades primarily provided sucrose, followed by malate (Figure 6c). Interestingly, in this condition, the old blades contributed more than the youngest blade to the provision of C at night, in stark contrast to warm days and nights where it is the opposite (Figure 6c). Unexpectedly, plants under 22 ºC:4 ºC presented a very irregular pattern of carbon consumption. Sheaths showed a slight sucrose accumulation during the night, and the third sheath provided malate. Blades still consumed more carbon than sheaths and relied mostly on sucrose and malate from the first and second blades, while the youngest blade consumed mainly starch (Figure 6b).

3.4 Cold nights affect mobilization of carbohydrates in *elf3* introgression lines without affecting photosynthesis and leaf elongation rates

The involvement of the circadian clock, particularly the *elf3*, in temperature responses and sugar metabolism has been described for Arabidopsis (Box *et al.*, 2015, Flis, Mengin, Ivakov, Mugford, Hubberten *et al.*, 2019). However, it has been proposed that the growth and C metabolism of monocots may not be affected on the same extent by circadian clock (Poire *et al.*, 2010). Therefore, to evaluate the effect of *elf3* on the partitioning of C compounds under cold in barley, Bowman WT and introgression lines 289 and 290 were grown under the three

growth conditions tested for Propino. When grown in 22°C:4°C, all genotypes took 20 days to reach same stage of development with third leaf developing and did not present differences in leaf elongation rates (Table 2). We measured net photosynthesis, stomatal conductance, internal concentration of CO_2, transpiration and water use efficiency (Table 3). No significant differences were observed between Bowman WT and *elf3* mutants. However, photosynthesis was decreased when cold day and nights were applied for all genotypes. Under only cold night condition, Propino showed a significantly decreased rate of photosynthesis compared to warm days and nights, other lines showing a non-significant tendency of decrease in photosynthesis rate (Table 3). The stomatal conductance and transpiration rate were increased under 10 ºC:4 ºC for all genotypes. This resulted in a lower WUE to all genotypes under 10 ºC:4 ºC (Table 3).

Primary metabolites were determined in blades and sheaths of WT and *elf3* introgression lines grown under 22 ºC:4 ºC at end of day and end of night period. As expected, cv. Bowman (WT) showed very similar metabolite patterns as those observed for Propino (Table S2).

Sheaths of third leaves contained more glucose than other tissues at end of day, and its content was lower in introgression lines than WT. Also introgression lines accumulated less glucose at end of day in the second sheath and blade (Table S2), which resulted in less C available for use at night. Fructose mostly accumulated in youngest sheath, and in all tissues its levels either remained stable or even increased during the night. No consistent significant differences were observed amongst genotypes for fructose levels except for the youngest leaf which contained less fructose at the end of the night and second and third sheaths at end of the day (Table S2). Sucrose was the second most accumulated C reserve in all genotypes, and the highest levels were found at end of day in the first and second blades. Most tissues showed partial consumption of sucrose at night in all genotypes (Table S2). Both introgression lines

presented slightly higher content of sucrose in first and second blades at end of night compared to WT (Table S2), an indication of less carbon consumed at night due to impaired function of *elf3*. Starch was not highly accumulated in blades or sheaths but was consumed at night in all tissues of all genotypes. Second and third blade accumulated more starch at end of day than other tissues in all genotypes under 22 ºC:4 ºC (Figure 7). However, introgression lines accumulated less starch at the end of day and also presented slightly higher level of starch than WT at end of night (Figure 7, Table S2). Blades of the first leaf accumulated more fructans than other tissues, with no significant difference between genotypes (Table S2). Despite low levels accumulated at end of day, mobilization of fructans was observed in the blade of first and second leaf for all genotypes. Malate was the most accumulated C compound in both blades and sheaths of all tissues and genotypes grown under 22ºC:4ºC. Malate was slightly mobilized in all blades at night in WT, but it was not observed in introgression lines (Table S2). Also malate levels were decreased in introgression lines in the first and second blades in the introgression lines, but increased the sheath of first leaf at end of the night, compared to WT. The proteins content was similar to all genotypes, although higher content was observed in blades of first and second leaves and third leaf parts with no significant mobilization at night (Table S2).

3.5 Crown has little participation in C supply for shoot growth under cold with incomplete C consumption in *elf3* introgression lines

To evaluate the contribution of the crown in the partition of carbohydrates and supply of growth under cold, we harvested 1 cm of crown tissue at end of day and end of night of all the genotypes grown under 22ºC:18ºC, 22 ºC:4 ºC and 10ºC:4ºC. The crown region comprises all meristematic tissues from which the apical meristem originates. The content of C compounds in crowns was much lower than in the shoot for all temperature conditions and genotypes, below 6 µmol g^{-1} FW for glucose, fructose and starch in all growth conditions.

Under warm days and nights, malate and fructans were the main metabolites with up to 20 and µmol g^{-1} FW, both compounds decreasing at night. Under warm days and cold nights, fructans and malate were again the main metabolites, with similar levels, but their levels did not decrease at night, and even increased for the fructans. Under cold days and nights, sucrose, fructans and malate were the major metabolites, reaching up to 26 µmol g^{-1} FW (Table S3). Protein content was very similar for all genotypes, with little variation between temperature conditions.

Under 22 ºC:18 ºC, Propino contained slightly more C compounds in crowns than Bowman WT. The *elf3* mutants presented similar levels of carbohydrates compared to WT, although lower levels of starch and malate. Under 22 ºC:4 ºC, only fructans at ED were lower than WT in *elf3* mutants. No consistent difference between WT and the *elf3* mutants were observed for all metabolites in crowns of plants grown under 10ºC:4ºC (Table S3).

4 DISCUSSION

4.1 Growth of barley is sensitive to both day and night cold

Barley reached three leaf stage at 15, 20 and 42 days when grown under 22ºC:18ºC, 22 ºC:4 ºC and 10ºC:4ºC, respectively. Thus, low temperatures both during the day and the night have a negative impact on the growth of young barley, which is in agreement with previous studies performed on barley and other monocotyledons (Poire *et al.*, 2010, Walter *et al.*, 2009). This is in stark contrast with Arabidopsis where growth is largely insensitive to cold night temperatures (Müller, Gol, Jeon, Weber, Davis *et al.*, 2018, Pyl, Piques, Ivakov, Schulze, Ishihara *et al.*, 2012). The maintenance of the growth in Arabidopsis was explained by an insensibility of starch degradation machinery to temperature, allowing C resources to be available even when temperature dropped, and an apparent excess in the growth machinery at optimal temperatures, via e.g. an incomplete mobilisation of the ribosomes for translation at warm temperatures, thus allowing the plants to mobilise this excess growth capacity when

temperature was dropping. Interestingly, in barley, the protein contents of all blades and sheaths, as well as the water content of the leaf 2 blade, were the same for both 22 ºC:18 ºC and 22 ºC:4 ºC temperature regimes (Figure 1a-b), in contrast to the plants grown under 10 ºC:4 ºC where protein levels were increased (Figure 1c). It suggests that cold night temperatures in barley, similar to Arabidopsis, do not lead to cold acclimation, which is characterised by an increase of the protein content (Guy, 1990, Pyl *et al.*, 2012) as well as an accumulation of sugars and other osmolytes (Alberdi & Corcuera, 1991, Bourion *et al.*, 2003, Trischuk *et al.*, 2014), thicker cell walls and then a lower water content (Gorsuch, Pandey & Atkin, 2010, Strand, Hurry, Henkes, Huner, Gustafsson *et al.*, 1999).

Thus, if the growth inhibition observed at night in barley (Figure 2) is not linked to extra costs incurred by elevated protein levels, it could be explained by (1) an inhibition of the transport of C resources from the source leaves to the sink leaves due to cold inhibition of phloem sap flow; (2) cold inhibition of the activities of enzymes involved in the degradation of C stores; and/or (3) cold inhibition of the activities of enzymes involved in the growth machinery. An inhibition of C transport from source to sink tissues is unlikely because cold nights mostly affected C mobilisation in the youngest growing blades, the consumption of C compounds during the night being the same for both temperature regimes in the oldest leaf (See Figure 3 for sucrose, Table S1 for all metabolites). Thus, an inhibition of the flow of phloem sap due to low temperatures leading to an unavailability of C for night growth is unlikely.

The second hypothesis is that cold driven inhibition of the activities of enzymes involved in the degradation of C stores explains the growth inhibition observed at night. In that case, we would expect only a partial degradation of the stores accumulated at ED, and potentially an increase in the C accumulated at ED if C assimilation in warm days was unaffected by cold nights. We observed a moderate inhibition of CO_2 assimilation for Propino plants growing under 22ºC:4ºC compared to 22ºC:18ºC, but Bowman and the *elf3* mutants did

not show significant changes (Table 3). The total amount of C accumulated at ED in 22ºC:4ºC was higher than at 22ºC:18ºC by around 20%, despite a major drop in starch content (43%), and was mostly explained by a major increase in malate content (around 1.7 fold). As well, we observed a decrease in the amount of C consumed under the 22ºC:4ºC and 10ºC:4ºC temperature regimes compared to 22ºC:18ºC. Finally, we observed that starch was still fully mobilised under cold nights (92%), similarly to Arabidopsis (Pyl et al., 2012), while sucrose and particularly malate, fructose, glucose and fructans percentages of mobilisation during cold nights were strongly reduced (Table 4). Thus a cold inhibition of the enzymes involved in the mobilisation of these compounds can at least partially explain the growth inhibition we observe.

Muller et al. (2018) hypothesised that the sensitivity of barley growth to cold nights is mostly due to sucrose mobilisation not being temperature compensated in contrast to starch mobilisation which is under clock control and temperature compensated. Their conclusions were based on data showing that starch was fully consumed at dawn but that large amounts of sucrose remained. We obtained qualitatively the same results at dawn for starch and sucrose levels. Moreover, when we calculated the percentages of consumption of both compounds during the night, we observed a maintenance of the starch mobilisation under the 22 ºC:4 ºC with 92% of the starch mobilised compared to 96% under 22ºC:18ºC, whilst mobilisation of sucrose was strongly depleted, from 96% under 22 ºC:18 ºC to 71% under 22 ºC:4 ºC (Table 4). However, if starch was largely consumed, its synthesis was also strongly inhibited and represented only 57% of the starch accumulated under the 22 ºC:18 ºC temperature regime. As a result, when we calculated the respective contribution of these metabolites to the overall C consumed at night, we did not observe any difference between the two growth conditions for sucrose, which contributed for ca 52% of the total carbon consumed at night, whilst starch contribution decreased from 26% to 18% (Table 4). Thus, the turnover of both compounds was

affected by low temperature, which suggests a tight regulation by barley of the night use of these two C stores. We conclude that cold nights affect both starch and sucrose metabolism, with starch synthesis and sucrose mobilisation being both repressed.

The third hypothesis to explain the inhibition of growth during cold nights is a temperature driven negative effect on the growth machinery. CBF genes, that are expressed under cold acclimation, control DELLA protein levels and gibberellin (GA) biosynthesis, resulting in a dwarf Arabidopsis phenotype by reducing GA synthesis, the overexpression of GA 2-oxidase and the accumulation of non-active forms of GA (Achard, Gong, Cheminant, Alioua, Hedden et al., 2008). Slender barley with defective DELLA is able to maintain its growth under cold, which is a phenotype that can be also mimicked by application of GA (Schünmann, Harrison & Ougham, 1994). Moreover, if the overexpression of Hv CBF2A reduces the time of cold acclimation required for acquiring freezing tolerance in barley, the transgenic plants were smaller than WT under normal growth conditions (Jeknic, Pillman, Dhillon, Skinner, Veisz et al., 2014). This could be partly circumvented by using stress induced specific promotors to modulate the expression of CBF genes (Yang, Al-Baidhani, Harris, Riboni, Li et al., 2019). Thus, growth under cold can be controlled independently of the availability of carbohydrates. However, how CBF and downstream genes do affect the growth machinery remains elusive. Moreover, how CBF genes could explain that cold nights do not affect growth in Arabidopsis but does in barley remains to be answered. Pyl et al. (2012) showed that in Arabidopsis rosettes, an increase of ribosome loading on mRNA in response to cold nights could provide a mechanism to compensate for the slower translational activity of ribosomes at low temperatures. That can only be possible if there is an excess of ribosomes for growth at warm temperatures. Interestingly, ribosomes can represent up to 30% of the total protein content of actively growing tissues in Arabidopsis, in contrast to ca 4% in a mature tissue (Sulpice, Ishihara, Schlereth, Cawthray, Encke et al., 2014). Strikingly, maize, which

like barley, is sensitive to low temperatures for night growth (Poire et al., 2010), does not show such a gradient of ribosome concentrations between the division and mature zones of an actively growing leaf (Czedik-Eysenberg, 2012), with only 1.6 times more ribosomes in the division zone than in the mature zone of a growing leaf. Therefore, we hypothesise that barley and maize might not have an excess in their growth machinery allowing them to compensate for environmental cues such as a drop in temperature. Sucrose and fructan accumulations in specific tissues are enhanced by cold during the light period, but not by cold nights.

Barley grown under the three temperature regimes did not show a major consumption of glucose and fructose in any tissue, with the exception of the youngest leaf sheath for plants grown under the 22 ºC:18 ºC regime (Figure S3 and S4). Rao *et al.* (2011) reported increases in glucose and fructose leaf pools followed by increase in fructans and sucrose when *Poa pratensis* was submitted to a cold acclimation treatment. However, after 8 days of acclimation at 5 ºC, there was no further increase in glucose or fructose levels. Our plants were grown in the three temperature regimes from sowing, so no accumulation of glucose or fructose were expected. Sucrose content was among the highest of all metabolites we determined at end of day in blades, which is agreement with previous studies (Gordon *et al.*, 1982, Gordon *et al.*, 1977, Gordon *et al.*, 1980a). However, sucrose levels in the sheaths were low for the three temperature regimes and this could be explained by significant high invertase activity in the sheaths (Roth *et al.*, 1997), also explaining the presence of glucose and fructose in the sheath of the young third leaf.

Fructan function has been largely associated to cold tolerance in plants of temperate regions (Abeynayake *et al.*, 2015, Morcuende, Kostadinova, Perez & Martinez-Carrasco, 2005, Tamura *et al.*, 2014, Tyrka *et al.*, 2015). In response to cold treatment, the expression of fructan synthesis genes is increased and consequently fructan levels rise (Meguro-Maoka & Yoshida, 2015, Morcuende *et al.*, 2005, Rao *et al.*, 2011, Tamura *et al.*, 2014, Yokota, Iehisa, Shimosaka

519 & Takumi, 2015). Accordingly, we would have expected increased content of fructans in plants
520 grown under the 22 ºC:4 ºC and 10 ºC:4 ºC temperature regimes. However, this was not the
521 case, and fructans were mostly observed in sheaths of youngest leaf 3 for all three temperature
522 regimes, with the highest levels being observed for the 10 ºC:4 ºC temperature regime,
523 representing less than 5% of the C accumulated at ED (Table 4).

524 Fructan synthesis is also stimulated by increases in sucrose content (Apolinario, de
525 Lima Damasceno, de Macedo Beltrao, Pessoa, Converti *et al.*, 2014, Arkel, 2014, Cairns, 2003,
526 Chalmers, Lidgett, Cummings, Cao, Forster *et al.*, 2005, Cimini, Locato, Vergauwen, Paradiso,
527 Cecchini *et al.*, 2015, Xue, Drenth, Glassop, Kooiker & McIntyre, 2013). In agreement,
528 fructans are synthesized particularly at the end of the day, when the diurnal levels of sucrose
529 are highest (Sicher, Kremer & Harris, 1984). This might explain the accumulation of fructans
530 in the oldest blade of the plants grown in 10 ºC:4 ºC temperature regime, as this leaf
531 accumulated the highest sucrose levels at end of the day, but it does not well explain why other
532 blades, which also accumulated high sucrose levels, did not accumulate fructans. It has been
533 proposed that fructan synthesis is induced only after a certain concentration of sucrose is
534 reached in the tissues, and that this level vary according to the species (Cairns, Cookson,
535 Thomas & Turner, 2002, Nagaraj *et al.*, 2004, Obenland, Simmen, Boller & Wiemken, 1991,
536 Suarez-Gonzalez, Lopez, Delano-Frier & Gomez-Leyva, 2014, Wagner & Wiemken, 1987,
537 Wagner, Wiemken & Matile, 1986). Whether sucrose accumulation was not enough to reach
538 the minimal level required to enhance fructan accumulation in other blades, or that fructan
539 accumulation is not naturally directly induced by sucrose due the different
540 compartmentalisation of sucrose and fructans (Cairns, Turner & Gallagher, 2008, Keerberg,
541 Ivanova, Keerberg, Parnik, Talts *et al.*, 2011) remains unclear. It is also possible that young
542 barley redirect sucrose towards growth instead of accumulating fructans even if temperature
543 drastically slows the development.

4.2 Starch accumulation is highly sensitive to cold nights, but not its mobilisation

Starch has been described as a minor reserve in fructan accumulating plants (Cairns *et al.*, 2002, Farrar & Farrar, 1985, Roth *et al.*, 1997, Wang & Tillberg, 1996, Wang, Van den Ende & Tillberg, 2000). However, our data show that starch is still an important transient carbon pool in young barley grown under 22 ºC:18 ºC (Table 4), representing 26% of the total C used during the night (Table 4). Conversely, we were surprised that starch accumulation was impaired (43% decrease) when the plants faced cold only at night (Figure 4c). Considering that the temperature in the light was the same in both conditions, the sucrose content at end of day was similar (Table 4), and that CO_2 assimilation was only slightly decreased (Table 3), we would expect no restrictions on starch accumulation. This result is in stark contrast with Pyl *et al.* (2012) who reported no change in starch accumulation and turnover in Arabidopsis plants submitted to cold nights. Starch turnover in Arabidopsis is strongly dependent on circadian clock (Graf *et al.*, 2010, Yazdanbakhsh, Sulpice, Graf, Stitt & Fisahn, 2011), and trehalose 6-phosphate (T6P) levels also regulate rates of starch degradation, allowing Arabidopsis plants to avoid exhaustion of starch prematurely during the night (Dos Anjos, Pandey, Moraes, Feil, Lunn *et al.*, 2018, Figueroa, Feil, Ishihara, Watanabe, Kolling *et al.*, 2016, Martins, Hejazi, Fettke, Steup, Feil *et al.*, 2013).

It has been proposed that the growth of both dicots and monocots is regulated by an additive effect of circadian-clock controlled processes and environmental cues such as temperature, with the monocots being more sensitive to environmental changes. We observed that in response to cold, either during 10 ºC:4 ºC or 22 ºC:4 ºC, the turnover of sucrose, glucose, fructose and malate were strongly reduced. But starch was still largely mobilised at night with 80-92% of it consumed under 22 ºC:4 ºC and 10 ºC:4 ºC compared to 96% for 22 ºC:18 ºC (Table 4). It appears that starch mobilisation is largely cold compensated in barley, like for Arabidopsis, but its accumulation during the day is impaired by night temperature. Thus, we

hypothesise that cold nights, which induce a strong reduction in growth of barley, inhibit starch accumulation in daytime (Figure 8). The mechanism in not known, but the circadian clock and/or T6P are likely candidates for such regulation and further studies are required. Both the clock and T6P signalling provide mechanisms for adjusting the rates of starch degradation (Graf *et al.*, 2010, Martins *et al.*, 2013), but recently they have been more largely involved in the diurnal control of both carbohydrate, organic acids and nitrogen metabolisms in Arabidopsis (Figueroa *et al.*, 2016, Flis *et al.*, 2019). Gordon, Ryle and Webb (1980b) suggested that starch consumption at night is triggered by a decrease of sucrose below a threshold value, rather than the onset of darkness. We also found a reduced rate of starch degradation in the first hours of the night for the 22 ºC:18 ºC treatment (Figure 3), so starch and sucrose use at night might be partly sequential. However our results show that if there is a threshold value for sucrose triggering starch degradation, then temperature do affect this value.

4.3 Malate plays important role as alternative carbon supply to growth

The levels of malate observed in barley leaves are very high. They are about 10 times more than in Arabidopsis rosettes grown at similar temperature and photoperiod (Medeiros, Barros, Barros, Omena-Garcia, Arrivault *et al.*, 2017). It is partly explained by Arabidopsis accumulating up to 10 μmol.g^{-1} FW of fumarate (Pracharoenwattana, Zhou, Keech, Francisco, Udomchalothorn *et al.*, 2010), in contrast to barley where fumarate was below detection levels (not shown). Malate is an intermediary of the tricarboxylic acid cycle (TCA) and it plays an important role in stomatal function, pH regulation and can refill the TCA cycle to restore NAD^+ and $NADP^+$ in the cell, besides being a major carbon storage in C_4 and CAM plants (Fernie & Martinoia, 2009). Plants that undergo chilling might increase reactive oxygen species (ROS) due to photo-inhibition (Allen & Ort, 2001, Hurry & Huner, 1992). Although malate could be a reductive equivalent complementing antioxidative mechanisms in presence of oxidative stress, the accumulation of malate in cold-hardened leaves of rye was probably about a storage

of carbon and a vacuolar osmolyte to balance cytosolic accumulation of sugars (Crecelius, Streb & Feierabend, 2003). The increased malate accumulation and significant turnover rates we observed in barley leaves under 22 ºC:4 ºC (Figure 5, Table 4) suggests that the malate pool might be an alternative carbon storage to starch and fructans, especially in young tissues (Figure 6). Malate contributed 15, 28 and 24% of the total carbon used at night for the 22ºC:18ºC, 22 ºC:4 ºC and 10 ºC:4 ºC temperature regimes, respectively (Table 4). It makes malate one of the major contributors of C for growth at night in barley. However, its turnover was only partial and high levels remain at dawn, especially under cold treatments, suggesting a role as an osmolyte in addition to a source of C for night use. Interestingly the highest levels of malate were in the youngest leaf, for all temperature regimes. Thus, malate might participate in the growth of young leaves, as well as in their protection, which was unexpected and strengthen the need for future metabolic studies in barley to include the determination of this compound.

4.4 Mobilization of C reserves is controlled by *elf3* but without an effect on growth under cold nights

Bowman WT and *elf3* mutants were grown under 22ºC:4ºC. Although there was no visual phenotype and mutants reached the same three leaf stage at same time as WT without differences in photosynthesis and elongation rates, we decided to investigate further if an impairment in *elf3* function caused disturbances in the regulation of C reserves. Glucose and fructose mobilization were affected in both *elf3* introgression lines at night, and importantly only a partial mobilisation of the starch and sucrose at night was observed (Table S2). While first and second blades showed a decrease in the consumption of sucrose at night in *elf3* mutants, the third sheaths of the mutants accumulated less hexoses at night compared to WT. These results suggest that in the *elf3* mutants, even if sucrose is only partially degraded in blades during the night and starch less accumulated during the day, it is partly compensated by

a decrease in the accumulation of hexoses during the night. These results exclude the possibility of growth impairment at night due to cold inhibition of phloem sap flow but supports the hypothesis of growth being sensitive to low temperature, as discussed above.

The participation of the crowns in the overall C accumulated at end of day, and the use of these reserves at night was very small compared to the shoots, because the C content accumulated in crowns was very low (Table S3). However, a small consumption for some of the carbohydrates (i.e. sucrose and starch) was observed, thus crowns act as transient reserve tissue. Crowns accumulate more fructans than the shoot parts at end of day, but they are not consumed during the night, instead, we see accumulation at end of night when cold night is imposed (Table S3). Accordingly, winter wheat shows decrease of almost 50% in assimilation rates under cold, but the ability to mobilize sucrose in leaves to fructans and starch in crowns allows higher assimilation rates than spring varieties (Savitch *et al.*, 2000). Interestingly, Vágújfalvi, Kerepesi, Galiba, Tischner and Sutka (1999) reported a significant correlation between accumulation of soluble carbohydrates in wheat varieties and freezing acclimation only after 19 days of treatment. Thus, the accumulation of fructans in spring varieties is limited at early stage, however, small increases of fructans in crown at night may play a role as osmolyte on the protection of young tissues from cold night and being mobilized during the day under warmer temperature.

5 CONCLUSION

Most carbon reserves used at night were stored in both young and mature blades and not in the sheaths, while crowns had little relevance in the accumulation of reserves for cold acclimation in young spring barley plants. Carbon consumed at night originated primarily from sucrose. However, malate was important, especially under cold treatments, and can be considered a major contributor to night growth in barley. Starch accumulation was strongly inhibited by cold in the daytime, but surprisingly also under warm day and cold night.

644 However, its mobilisation was not affected by cold nights. The clock *elf3* mutants showed
645 changes in glucose, fructose, sucrose and starch levels compared to WT, however, it did not
646 impact on their growth, maybe because these metabolites were not drastically affected
647 quantitatively. Altogether our data suggest that enzymes involved in the mobilisation of sucrose
648 and malate and/or barley growth machinery are sensitive to cold night because C was available
649 for night growth and despite that, the plants were not growing. Thus, breeding for increased
650 photosynthetic performance under cold might not lead to increases in biomass in barley because
651 it is the use of the photosynthates which is limiting. In contrast, desensitising growth inhibition
652 by cold might be an alternative target, through e.g. higher polysome recruitment, manipulation
653 of the CBF pathway and the regulation of DELLA proteins.

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REFERENCES

Abeynayake S.W., Etzerodt T.P., Jonaviciene K., Byrne S., Asp T. & Boelt B. (2015) Fructan metabolism and changes in fructan composition during cold acclimation in perennial ryegrass. *Frontiers in Plant Science*, **6**, 329.

Achard P., Gong F., Cheminant S., Alioua M., Hedden P. & Genschik P. (2008) The cold-inducible CBF1 factor-dependent signaling pathway modulates the accumulation of the growth-repressing DELLA proteins via its effect on gibberellin metabolism. *Plant Cell*, **20**, 2117-2129.

Al-Hamdani S.H. & Thomas T.S. (2001) Influence of root chilling on winter and spring wheat growth and carbon dioxide assimilation. *Acta Agriculturae Scandinavica Section B-Soil and Plant Science*, **50**, 149-154.

Alberdi M. & Corcuera L.J. (1991) Cold-acclimation in plants. *Phytochemistry*, **30**, 3177-3184.

Allen D.J. & Ort D.R. (2001) Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends in Plant Science*, **6**, 36-42.

Apolinario A.C., de Lima Damasceno B.P., de Macedo Beltrao N.E., Pessoa A., Converti A. & da Silva J.A. (2014) Inulin-type fructans: a review on different aspects of biochemical and pharmaceutical technology. *Carbohydrate Polymers*, **101**, 368-378.

Arkel V. (2014) Fructan biosynthesis regulation and the production of tailor-made fructan in plants. *Polysaccharides: Natural Fibers in Food and Nutrition*, 1-29.

Bourion V., Lejeune-Henaut I., Munier-Jolain N. & Salon C. (2003) Cold acclimation of winter and spring peas: carbon partitioning as affected by light intensity. *European Journal of Agronomy*, **19**, 535-548.

Box M.S., Huang B.E., Domijan M., Jaeger K.E., Khattak A.K., Yoo S.J., Sedivy E.L., Jones D.M., Hearn T.J., Webb A.A.R., Grant A., Locke J.C.W. & Wigge P.A. (2015) ELF3 controls thermoresponsive growth in Arabidopsis. *Current Biology*, **25**, 194-199.

Cairns A.J. (2003) Fructan biosynthesis in transgenic plants. *Journal of Experimental Botany*, **54**, 549-567.

Cairns A.J., Cookson A., Thomas B.J. & Turner L.B. (2002) Starch metabolism in the fructan-grasses: patterns of starch accumulation in excised leaves of *Lolium temulentum* L. *Journal of Plant Physiology*, **159**, 293-305.

Cairns A.J., Turner L.B. & Gallagher J.A. (2008) Ryegrass leaf fructan synthesis is oxygen dependent and abolished by endomembrane inhibitors. *New Phytologist*, **180**, 832-840.

Chalmers J., Lidgett A., Cummings N., Cao Y., Forster J. & Spangenberg G. (2005) Molecular genetics of fructan metabolism in perennial ryegrass. *Plant Biotechnology Journal*, **3**, 459-474.

Cimini S., Locato V., Vergauwen R., Paradiso A., Cecchini C., Vandenpoel L., Verspreet J., Courtin C.M., D'Egidio M.G., Van den Ende W. & De Gara L. (2015) Fructan biosynthesis and degradation as part of plant metabolism controlling sugar fluxes during durum wheat kernel maturation. *Frontiers in Plant Science*, **6**, 89.

Conry M.J. (1995) Comparison of early, normal and late sowing at three rates of nitrogen on the yield, grain nitrogen and screenings content of Blenheim spring malting barley in Ireland. *Journal of Agricultural Science*, **125**, 183-188.

Conry M.J. (1998) Influence of seed rate and sowing date on the yield and grain quality of Blenheim spring malting barley in the south-east of Ireland. *Journal of Agricultural Science*, **130**, 307-315.

Cook D., Fowler S., Fiehn O. & Thomashow M.F. (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 15243-15248.

Crecelius F., Streb P. & Feierabend J. (2003) Malate metabolism and reactions of oxidoreduction in cold-hardened winter rye (*Secale cereale* L.) leaves. *Journal of Experimental Botany*, **54**, 1075-1083.

Czedik-Eysenberg A. (2012) *Elucidating connections between metabolism and growth in Zea mays*.

del Viso F., Puebla A.F., Fusari C.M., Casabuono A.C., Couto A.S., Pontis H.G., Hopp H.E. & Heinz R.A. (2009) Molecular characterization of a putative sucrose: Fructan-6-fructosyltransferase (6-SFT) of the cold-resistant patagonian grass *Bromus pictus* associated with fructan accumulation under low temperatures. *Plant and Cell Physiology*, **50**, 489-503.

Dos Anjos L., Pandey P.K., Moraes T.A., Feil R., Lunn J.E. & Stitt M. (2018) Feedback regulation by trehalose 6-phosphate slows down starch mobilization below the rate that would exhaust starch reserves at dawn in Arabidopsis leaves. *Plant Direct*, **2**, e00078.

Espinoza C., Degenkolbe T., Caldana C., Zuther E., Leisse A., Willmitzer L., Hincha D.K. & Hannah M.A. (2010) Interaction with Diurnal and Circadian Regulation Results in Dynamic Metabolic and Transcriptional Changes during Cold Acclimation in Arabidopsis. *Plos One*, **5**.

Farrar S.C. & Farrar J.F. (1985) Carbon fluxes in leaf blades of barley. *New Phytologist*, **100**, 271-283.

Faure S., Turner A.S., Gruszka D., Christodoulou V., Davis S.J., von Korff M. & Laurie D.A. (2012) Mutation at the circadian clock gene *EARLY MATURITY 8* adapts domesticated barley (*Hordeum vulgare*) to short growing seasons. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 8328-8333.

Fernie A.R. & Martinoia E. (2009) Malate. Jack of all trades or master of a few? *Phytochemistry*, **70**, 828-832.

Figueroa C.M., Feil R., Ishihara H., Watanabe M., Kolling K., Krause U., Hohne M., Encke B., Plaxton W.C., Zeeman S.C., Li Z., Schulze W.X., Hoefgen R., Stitt M. & Lunn J.E. (2016) Trehalose 6-phosphate coordinates organic and amino acid metabolism with carbon availability. *Plant Journal*, **85**, 410-423.

Flis A., Mengin V., Ivakov A.A., Mugford S.T., Hubberten H.M., Encke B., Krohn N., Hohne M., Feil R., Hoefgen R., Lunn J.E., Millar A.J., Smith A.M., Sulpice R. & Stitt M. (2019) Multiple circadian clock outputs regulate diel turnover of carbon and nitrogen reserves. *Plant Cell and Environment*, **42**, 549-573.

Ford B., Deng W.W., Clausen J., Oliver S., Boden S., Hemming M. & Trevaskis B. (2016) Barley (*Hordeum vulgare*) circadian clock genes can respond rapidly to temperature in an *EARLY FLOWERING 3*-dependent manner. *Journal of Experimental Botany*, **67**, 5517-5528.

Genty B., Briantais J.-M. & Baker N.R. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Bioch et Biophys Acta*, **990**, 87-92.

Gordon A.J., Ryle G.J.A., Mitchell D.F. & Powell C.E. (1982) The dynamics of carbon supply from leaves of barley plants grown in long or short days. *Journal of Experimental Botany*, **33**, 241-250.

Gordon A.J., Ryle G.J.A. & Powell C.E. (1977) The Strategy of Carbon Utilization in Uniculm Barley I. THE CHEMICAL FATE OF PHOTOSYNTHETICALLY ASSIMILATED14C. *Journal of Experimental Botany*, **28**, 1258-1269.

Gordon A.J., Ryle G.J.A. & Powell C.E. (1979) The strategy of carbon utilization in uniculm barley II. The effect of continuous light and continuous dark

treatments. *Journal of Experimental Botany*, **30**, 589-599.

Gordon A.J., Ryle G.J.A., Powell C.E. & Mitchell D. (1980a) Export, Mobilization, and Respiration of Assimilates in Uniculm Barley during Light and Darkness. *Journal of Experimental Botany*, **31**, 461-473.

Gordon A.J., Ryle G.J.A. & Webb G. (1980b) The Relationship between Sucrose and Starch during 'Dark' Export from Leaves of Uniculm Barley. *Journal of Experimental Botany*, **31**, 845-850.

Gorsuch P.A., Pandey S. & Atkin O.K. (2010) Temporal heterogeneity of cold acclimation phenotypes in *Arabidopsis* leaves. *Plant Cell & Environment*, **33**, 244-258.

Graf A., Schlereth A., Stitt M. & Smith A.M. (2010) Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 9458-9463.

Guy C.L. (1990) Cold-acclimation and freezing stress tolerance - role of protein-metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology*, **41**, 187-223.

Hendriks J.H.M., Kolbe A., Gibon Y., Stitt M. & Geigenberger P. (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. *Plant Physiology*, **133**, 838.

Hurry V.M. & Huner N.P.A. (1992) Effect of cold hardening on sensitivity of winter and spring wheat leaves to short-term photoinhibition and recovery of photosynthesis. *Plant Physiology*, **100**, 1283-1290.

Hurry V.M., Strand A., Tobiaeson M., Gardestrom P. & Oquist G. (1995) Cold hardening of spring and winter-wheat and rape results in differential-effects on growth, carbon metabolism, and carbohydrate content. *Plant Physiology*, **109**, 697-706.

Janmohammadi M., Mock H.P. & Matros A. (2014) Proteomic analysis of cold acclimation in winter wheat under field conditions. *Icelandic Agricultural Sciences*, **27**, 3-15.

Jeknic Z., Pillman K.A., Dhillon T., Skinner J.S., Veisz O., Cuesta-Marcos A., Hayes P.M., Jacobs A.K., Chen T.H.H. & Stockinger E.J. (2014) Hv-CBF2A overexpression in barley accelerates COR gene transcript accumulation and acquisition of freezing tolerance during cold acclimation. *Plant Molecular Biology*, **84**, 67-82.

Jeong B.R. & Housley T.L. (1990) Fructan metabolism in wheat in alternating warm and cold temperatures. *Plant Physiology*, **93**, 902-906.

Jin Y., Fei M., Rosenquist S., Jin L., Gohil S., Sandstrom C., Olsson H., Persson C., Hoglund A.S., Fransson G., Ruan Y., Aman P., Jansson C., Liu C., Andersson R. & Sun C. (2017) A dual-promoter gene orchestrates the sucrose-coordinated synthesis of starch and fructan in barley. *Molecular Plant*, **10**, 1556-1570.

Keerberg O., Ivanova H., Keerberg H., Parnik T., Talts P. & Gardestrom P. (2011) Quantitative analysis of photosynthetic carbon metabolism in protoplasts and intact leaves of barley. Determination of carbon fluxes and pool sizes of metabolites in different cellular compartments. *BioSystems*, **103**, 291-301.

Kirby E.J.M., Appleyard M. & Fellowes G. (1982) Effect of Sowing Date on the Temperature Response of Leaf Emergence and Leaf Size in Barley. *Plant Cell and Environment*, **5**, 477-484.

Kirby E.J.M., Appleyard M. & Fellowes G. (1985) Variation in Development of Wheat and Barley in Response to Sowing Date and Variety. *Journal of Agricultural Science*, **104**, 383-396.

Kitajima M. & Butler W.L. (1975) Quenching of Chlorophyll Fluorescence and Primary Photochemistry in Chloroplasts by Dibromothymoquinone. *Biochimica Et Biophysica Acta*, **376**, 105-115.

Krapp A., Hofmann B., Schafer C. & Stitt M. (1993) Regulation of the expression of rbcs and other photosynthetic genes by carbohydrates - a mechanism for the sink regulation of photosynthesis. *Plant Journal*, **3**, 817-828.

Kumar P., Singh B., Hooda V.S., Sewhag M. & Chaudhary A. (2017) Effect of different dates of sowing on yield attributes, yield and quality of Barley (Hordeum vulgare L.) cultivars. *Journal of Applied and Natural Science*, **9**, 129-132.

Lorenzo M., Assuero S.G. & Tognetti J.A. (2015) Low temperature differentially affects tillering in spring and winter wheat in association with changes in plant carbon status. *Annals of Applied Biology*, **166**, 236-248.

Ludewig F. & Sonnewald U. (2016) Demand for food as driver for plant sink development. *Journal of Plant Physiology*, **203**, 110-115.

Martins M.C.M., Hejazi M., Fettke J., Steup M., Feil R., Krause U., Arrivault S., Vosloh D., Figueroa C.M., Ivakov A., Yadav U.P., Piques M., Metzner D., Stitt M. & Lunn J.E. (2013) Feedback Inhibition of Starch Degradation in Arabidopsis Leaves Mediated by Trehalose 6-Phosphate. *Plant Physiology*, **163**, 1142-1163.

Medeiros D.B., Barros K., Barros J.A., Omena-Garcia R.P., Arrivault S., Vincis Pereira Sanglard L., Detmann K.C., Silva W.B., Daloso D.M., DaMatta F., Nunes-Nesi A., Fernie A.R. & Araújo W.L. (2017) Impaired malate and fumarate accumulation due the mutation of tonoplast dicarboxylate transporter. *Plant Physiology*.

Meguro-Maoka A. & Yoshida M. (2015) Analysis of seasonal expression levels of wheat fructan exohydrolase (FEH) genes regulating fructan metabolism involved in wintering ability. *Journal of Plant Physiology*, **191**, 54-62.

Morcuende R., Kostadinova S., Perez P. & Martinez-Carrasco R. (2005) Fructan synthesis is inhibited by phosphate in warm-grown, but not in cold-treated, excised barley leaves. *New Phytologist*, **168**, 567-574.

Müller L.M., Gol L., Jeon J.-S., Weber A.P.M., Davis S.J. & von Korff M. (2018) Temperature but not the circadian clock determines nocturnal carbohydrate availability for growth in cereals. *bioRxiv*.

Müller L.M., von Korff M. & Davis S.J. (2014) Connections between circadian clocks and carbon metabolism reveal species-specific effects on growth control. *Journal of Experimental Botany*, **65**, 2915-2923.

Nagaraj V.J., Altenbach D., Galati V., Luscher M., Meyer A.D., Boller T. & Wiemken A. (2004) Distinct regulation of sucrose: sucrose-1-fructosyltransferase (1-SST) and sucrose: fructan-6-fructosyltransferase (6-SFT), the key enzymes of fructan synthesis in barley leaves: 1-SST as the pacemaker. *New Phytologist*, **161**, 735-748.

Nagaraj V.J., Riedl R., Boller T., Wiemken A. & Meyer A.D. (2001) Light and sugar regulation of the barley sucrose : fructan 6-fructosyltransferase promoter. *Journal of Plant Physiology*, **158**, 1601-1607.

Obenland D.M., Simmen U., Boller T. & Wiemken A. (1991) Regulation of sucrose-sucrose-fructosyltransferase in barley leaves. *Plant Physiology*, **97**, 811-813.

Oquist G., Hurry V.M. & Huner N.P.A. (1993) Low-Temperature Effects on Photosynthesis and Correlation with Freezing Tolerance in Spring and Winter Cultivars of Wheat and Rye. *Plant Physiology*, **101**, 245-250.

Pare S., Gilmour S.J., Grumet R. & Thomashow M.F. (2018) CBF-dependent and CBF-independent regulatory pathways contribute to the differences in freezing tolerance and cold-regulated gene expression of two Arabidopsis ecotypes locally adapted to sites in Sweden and Italy. *Plos One*, **13**.

Photiades I. & Hadjichristodoulou A. (1984) Sowing Date, Sowing Depth, Seed Rate and Row Spacing of Wheat and Barley under Dryland Conditions. *Field Crops Research*, **9**, 151-162.

Poire R., Wiese-Klinkenberg A., Parent B., Mielewczik M., Schurr U., Tardieu F. & Walter A. (2010) Diel time-courses of leaf growth in monocot and dicot species: endogenous rhythms and temperature effects. *Journal of Experimental Botany*, **61**, 1751-1759.

Pommerrenig B., Ludewig F., Cvetkovic J., Trentmann O., Klemens P.A.W. & Neuhaus H.E. (2018) In concert: Orchestrated changes in carbohydrate homeostasis are critical for plant abiotic stress tolerance. *Plant and Cell Physiology*, **59**, 1290-1299.

Potterton E.M. & McCabe T. (2018) The effect of sowing date and nitrogen rate on the grain yield, grain quality and malt analyses of spring malting barley for distilling in Ireland. *Journal of Agricultural Science*, **156**, 515-527.

Pracharoenwattana I., Zhou W.X., Keech O., Francisco P.B., Udomchalothorn T., Tschoep H., Stitt M., Gibon Y. & Smith S.M. (2010) Arabidopsis has a cytosolic fumarase required for the massive allocation of photosynthate into fumaric acid and for rapid plant growth on high nitrogen. *Plant Journal*, **62**, 785-795.

Pyl E.T., Piques M., Ivakov A., Schulze W., Ishihara H., Stitt M. & Sulpice R. (2012) Metabolism and growth in Arabidopsis depend on the daytime temperature but are temperature-compensated against cool nights. *Plant Cell*, **24**, 2443-2469.

Rao R.S.P., Andersen J.R., Dionisio G. & Boelt B. (2011) Fructan accumulation and transcription of candidate genes during cold acclimation in three varieties of *Poa pratensis*. *Journal of Plant Physiology*, **168**, 344-351.

Ritsema T., Brodmann D., Diks S.H., Bos C.L., Nagaraj V., Pieterse C.M., Boller T., Wiemken A. & Peppelenbosch M.P. (2009) Are small GTPases signal hubs in sugar-mediated induction of fructan biosynthesis? *PLoS One*, **4**, e6605.

Roth A., Luscher M., Sprenger N., Boller T. & Wiemken A. (1997) Fructan and fructan-metabolizing enzymes in the growth zone of barley leaves. *New Phytologist*, **136**, 73-79.

Ruelland E., Vaultier M.N., Zachowski A. & Hurry V. (2009) Cold signalling and cold acclimation in plants. *Advances in Botanical Research, Vol 49*, **49**, 35-150.

Savitch L.V., Harney T. & Huner N.P.A. (2000) Sucrose metabolism in spring and winter wheat in response to high irradiance, cold stress and cold acclimation. *Physiologia Plantarum*, **108**, 270-278.

Schnyder H. (1993) The role of carbohydrate storage and redistribution in the source-sink relations of wheat and barley during grain filling - a review. *New Phytologist*, **123**, 233-245.

Schünmann P.H.D., Harrison J. & Ougham H.J. (1994) Slender barley, an extension growth mutant. *Journal of Experimental Botany*, **45**, 1753-1760.

Shi Y., Ding Y. & Yang S. (2018) Molecular regulation of CBF signaling in cold acclimation. *Trends in Plant Science*, **23**, 623-637.

Sicher R.C., Kremer D.F. & Harris W.G. (1984) Diurnal carbohydrate-metabolism of barley primary leaves. *Plant Physiology*, **76**, 165-169.

Smouter H. & Simpson R.J. (1991) Fructan metabolism in leaves of *Lolium rigidum* Gaudin. *New Phytologist*, **119**, 509-516.

Stitt M., Vonschaewen A. & Willmitzer L. (1991) Sink regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell-wall

involves a decrease of the Calvin-cycle enzymes and an increase of glycolytic-enzymes. *Planta*, **183**, 40-50.

Strand A., Hurry V., Henkes S., Huner N., Gustafsson P., Gardestrom P. & Stitt M. (1999) Acclimation of Arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. *Plant Physiology*, **119**, 1387-1397.

Suarez-Gonzalez E.M., Lopez M.G., Delano-Frier J.P. & Gomez-Leyva J.F. (2014) Expression of the 1-SST and 1-FFT genes and consequent fructan accumulation in *Agave tequilana* and *A. inaequidens* is differentially induced by diverse (a)biotic-stress related elicitors. *Journal of Plant Physiology*, **171**, 359-372.

Sulpice R., Ishihara H., Schlereth A., Cawthray G.R., Encke B., Giavalisco P., Ivakov A., Arrivault S., Jost R., Krohn N., Kuo J., Laliberte E., Pearse S.J., Raven J.A., Scheible W.R., Teste F., Veneklaas E.J., Stitt M. & Lambers H. (2014) Low levels of ribosomal RNA partly account for the very high photosynthetic phosphorus-use efficiency of Proteaceae species. *Plant Cell Environ*, **37**, 1276-1298.

Tamura K., Sanada Y., Tase K. & Yoshida M. (2014) Fructan metabolism and expression of genes coding fructan metabolic enzymes during cold acclimation and overwintering in timothy (*Phleum pratense*). *Journal of Plant Physiology*, **171**, 951-958.

Tarkowski L.P. & Van den Ende W. (2015) Cold tolerance triggered by soluble sugars: a multifaceted countermeasure. *Frontiers in Plant Science*, **6**, 203.

Thomashow M.F. (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology*, **50**, 571-599.

Thomashow M.F. (2010) Molecular basis of plant cold acclimation: Insights gained from studying the CBF cold response pathway. *Plant Physiology*, **154**, 571-577.

Trischuk R.G., Schilling B.S., Low N.H., Gray G.R. & Gusta L.V. (2014) Cold acclimation, de-acclimation and re-acclimation of spring canola, winter canola and winter wheat: The role of carbohydrates, cold-induced stress proteins and vernalization. *Environmental and Experimental Botany*, **106**, 156-163.

Tyrka M., Rapacz M., Fiust A., Wójcik-Jagła M. & Rognli O.A. (2015) Quantitative trait loci mapping of freezing tolerance and photosynthetic acclimation to cold in winter two- and six-rowed barley. *Plant Breeding*, **134**, 271-282.

Vágújfalvi A., Kerepesi I., Galiba G., Tischner T. & Sutka J. (1999) Frost hardiness depending on carbohydrate changes during cold acclimation in wheat. *Plant Science*, **144**, 85-92.

Visioni A., Tondelli A., Francia E., Pswarayi A., Malosetti M., Russell J., Thomas W., Waugh R., Pecchioni N., Romagosa I. & Comadran J. (2013) Genome-wide association mapping of frost tolerance in barley (Hordeum vulgare L.). *BMC Genomics*, **14**, 424.

Wagner W. & Wiemken A. (1987) Enzymology of fructan synthesis in grasses: Properties of sucrose-sucrose-fructosyltransferase in barley leaves (*Hordeum-vulgare*-L cv Gerbel). *Plant Physiology*, **85**, 706-710.

Wagner W., Wiemken A. & Matile P. (1986) Regulation of fructan metabolism in leaves of barley (*Hordeum vulgare* L cv Gerbel). *Plant Physiology*, **81**, 444-447.

Walter A., Silk W.K. & Schurr U. (2009) Environmental effects on spatial and temporal patterns of leaf and root growth. *Annual Review of Plant Biology*, **60**, 279-304.

Wang C.W. & Tillberg J.E. (1996) Effects of nitrogen deficiency on accumulation of fructan and fructan metabolizing enzyme activities in sink and source leaves of barley (*Hordeum vulgare*). *Physiologia Plantarum*, **97**, 339-345.

Wang C.W., Van den Ende W. & Tillberg J.E. (2000) Fructan accumulation induced by nitrogen deficiency in barley leaves correlates with the level of sucrose : fructan 6-fructosyltransferase mRNA. *Planta*, **211**, 701-707.

Wang H., Yan S.J., Xin H.J., Huang W.J., Zhang H., Teng S.Z., Yu Y.C., Fernie A.R., Lu X.D., Li P.C., Li S.Y., Zhang C.Y., Ruan Y.L., Chen L.Q. & Lang Z.H. (2019) A Subsidiary Cell-Localized Glucose Transporter Promotes Stomatal Conductance and Photosynthesis. *Plant Cell*, **31**, 1328-1343.

Xue G.P., Drenth J., Glassop D., Kooiker M. & McIntyre C.L. (2013) Dissecting the molecular basis of the contribution of source strength to high fructan accumulation in wheat. *Plant Molecular Biology*, **81**, 71-92.

Yang Y.F., Al-Baidhani H.H.J., Harris J., Riboni M., Li Y., Mazonka I., Bazanovas N., Chirkova L., Hussain S.S., Hrmova M., Haefele S., Lopato S. & Kovalchuk N. (2019) DREB/CBF expression in wheat and barley using the stress-inducible promoters of HD-Zip I genes: impact on plant development, stress tolerance and yield. *Plant Biotechnology Journal*.

Yazdanbakhsh N., Sulpice R., Graf A., Stitt M. & Fisahn J. (2011) Circadian control of root elongation and C partitioning in *Arabidopsis thaliana*. *Plant Cell & Environment*, **34**, 877-894.

Yokota H., Iehisa J.C.M., Shimosaka E. & Takumi S. (2015) Line differences in Cor/Lea and fructan biosynthesis-related gene transcript accumulation are related to distinct freezing tolerance levels in synthetic wheat hexaploids. *Journal of Plant Physiology*, **176**, 78-88.

Table 1. Leaf 2 water content and fluorescence parameters of Propino cv. grown under different temperature regimes.

	Water content (%)	F_v/F_m	$Y_{(II)}$	ETR
22ºC:18ºC	91.15 ± 1.11 a	0.796 ± 0.003 a	0.745±0.016 a	156.4±3.4 a
22ºC:4ºC	88.97 ± 0.92 b	0.788 ± 0.006 a	0.743±0.009 a	156.1±1.9 a
10ºC:4ºC	84.59 ± 0.96 c	0.77 ± 0.016 b	0.722±0.009 b	151.5±1.9 b

Propino plants were grown until third leaf stage in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. The plants reached third leaf stage under 22°C:18°C day:night at 15 DAS, 22°C:4°C day:night at 20 DAS and 10°C:4°C day:night at 42 DAS. Values represent mean and SD. F_v/Fm: maximum photochemical quantum yield of PS II; $Y_{(II)}$: effective photochemical quantum yield of PS II; ETR: electron transport rate in µmol electron $m^{-2}s^{-1}$. Letters represent significant differences between treatments for Tukey's test $P<0.05$, n=6.

Table 2. Elongation rate and height of Bowman WT and *elf3* introgression lines under cold nights.

Elongation rate (mm h^{-1})			
Second leaf	**WT**	**289**	**290**
Day	1.9 ± 0.2 a*	2.1 ± 0.2 a*	2.0 ± 0.2 a*
Night	0.4 ± 0.2 a	0.3 ± 0.1 a	0.3 ± 0.1 a
Third leaf	**WT**	**289**	**290**
Day	1.9 ± 0.1 a*	1.9 ± 0.1 a*	2.0 ± 0.2 a*
Night	0.4 ± 0.1 a	0.4 ± 0.0 a	0.2 ± 0.1 a
Height (cm)			
WT	20.0 ± 2.0 a		
289	20.7 ± 1.3 a		
290	18.7 ± 1.3 a		

Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ 22°C:4°C day:night for 20 DAS, until third leaf stage Values represent mean and SD. Letters represent differences between genotypes by Tukey $P<0.05$; * represents differences between day and night by t-test $P<0.05$, n=6.

Table 3. Photosynthesis, stomatal conductance, internal CO_2 concentration, transpiration and water use efficiency of Bowman (WT), *elf3* mutants and Propino, grown under different temperature regimes

		A		g_s		C_i		E		WUE	
22ºC:18ºC	**WT**	17035 ± 1817	B	0.004 ± 0.001	A	22 ± 5	A	0.043 ± 0.011	A	112 ± 30	B
	289	17354 ± 2869	B	0.005 ± 0.001	A	22 ± 6	A	0.056 ± 0.007	A	91 ± 18	B
	290	16516 ± 1373	B	0.004 ± 0.001	A	21 ± 6	A	0.043 ± 0.012	A	111 ± 31	B
	PRO	17567 ± 3149	B	0.004 ± 0.000	A	20 ± 5	A	0.050 ± 0.011	A	110 ± 15	C
22ºC:4ºC	**WT**	15674 ± 2168	B	0.004 ± 0.001	A	20 ± 2	A	0.053 ± 0.011	A	88 ± 16	B
	289	15266 ± 1213	B	0.005 ± 0.001	A	19 ± 2	A	0.055 ± 0.009	A	80 ± 14	B
	290	14781 ± 1514	B	0.004 ± 0.001	A	22 ± 5	A	0.048 ± 0.011	A	92 ± 15	B
	PRO	13150 ± 1290	A	0.004 ± 0.001	A	18 ± 3	A	0.045 ± 0.006	A	87 ± 17	B
10ºC:4ºC	**WT**	11498 ± 1175	A	0.016 ± 0.006	B	22 ± 3	Ab	0.080 ± 0.019	B	18 ± 6	A
	289	10920 ± 1764	A	0.018 ± 0.006	B	21 ± 2	Aab	0.081 ± 0.022	B	16 ± 6	A
	290	11012 ± 1334	A	0.014 ± 0.003	B	23 ± 4	Ab	0.073 ± 0.010	B	18 ± 4	A
	PRO	10601 ± 983	A	0.013 ± 0.004	B	16 ± 3	Aa	0.060 ± 0.015	A	20 ± 4	A

Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C day:night for 15 DAS, 22°C:4°C day:night for 20 DAS and 10°C:4°C day:night for 45 DAS, until third leaf stage. Values represent mean and SD. WT: Bowman WT; 289 and 290: introgression lines for *elf3* in Bowman background; PRO: cv. Propino; A: net photosynthesis, µmol g^{-1} DW day^{-1}; g_s: stomatal conductance, mol H_2O g^{-1} DW day^{-1}; C_i: substomatal concentration of CO_2; E: transpiration, $mol.g^{-1}.day^{-1}$; WUE: water use efficiency µmol CO_2 mol^{-1} H_2O. Capital

letters represent differences between temperature regime within a genotype; small case letters represent differences between genotypes within temperature regimes by Tukey $P<0.05$, n=6.

Table 4. Accumulation and mobilization of reserves in barley grown under three temperature regimes.

Total accumulation at end of day (C µmol g-1 FW)			
	22ºC:18ºC	**22ºC:4ºC**	**10ºC:4ºC**
Glucose	11 ± 1 b	13 ± 1 b	8 ± 1 a
Fructose	4 ± 0 b	3 ± 0 a	4 ± 0 b
Sucrose	159 ± 14 a	174 ± 7 a	181 ± 8 a
Starch	82 ± 3 c	46 ± 4 b	27 ± 0 a
Fructans	7 ± 5 a	6 ± 1 a	13 ± 0 a
Malate	52 ± 3 c	127 ± 14 a	89 ± 8 b
Total[1]	316 ± 10 a	370 ± 21 b	321 ± 6 a
Depletion of reserves during the night [%]			
	22ºC:18ºC	**22ºC:4ºC**	**10ºC:4ºC**
Glucose	94	30	46
Fructose	97	-85	-1
Sucrose	96	71	83
Starch	96	92	80
Fructans	93	47	55
Malate	84	53	64
Total[2]	94	64	74
Contribution to C use at night [%]			
	22ºC:18ºC	**22ºC:4ºC**	**10ºC:4ºC**
Glucose	4	2	2
Fructose	1	-1	0
Sucrose	52	52	63
Starch	26	18	9
Fructans	2	1	3
Malate	15	28	24

Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C day:night for 15 DAS, 22°C:4°C day:night for 20 DAS and 10°C:4°C day:night

for 42 DAS, until third leaf stage. Values represent mean and SD. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. n= 3

*Total of reserves accumulated at end of day in the shoot

**Percentage of the total reserves consumed at night in shoots from total reserves accumulated at end of day

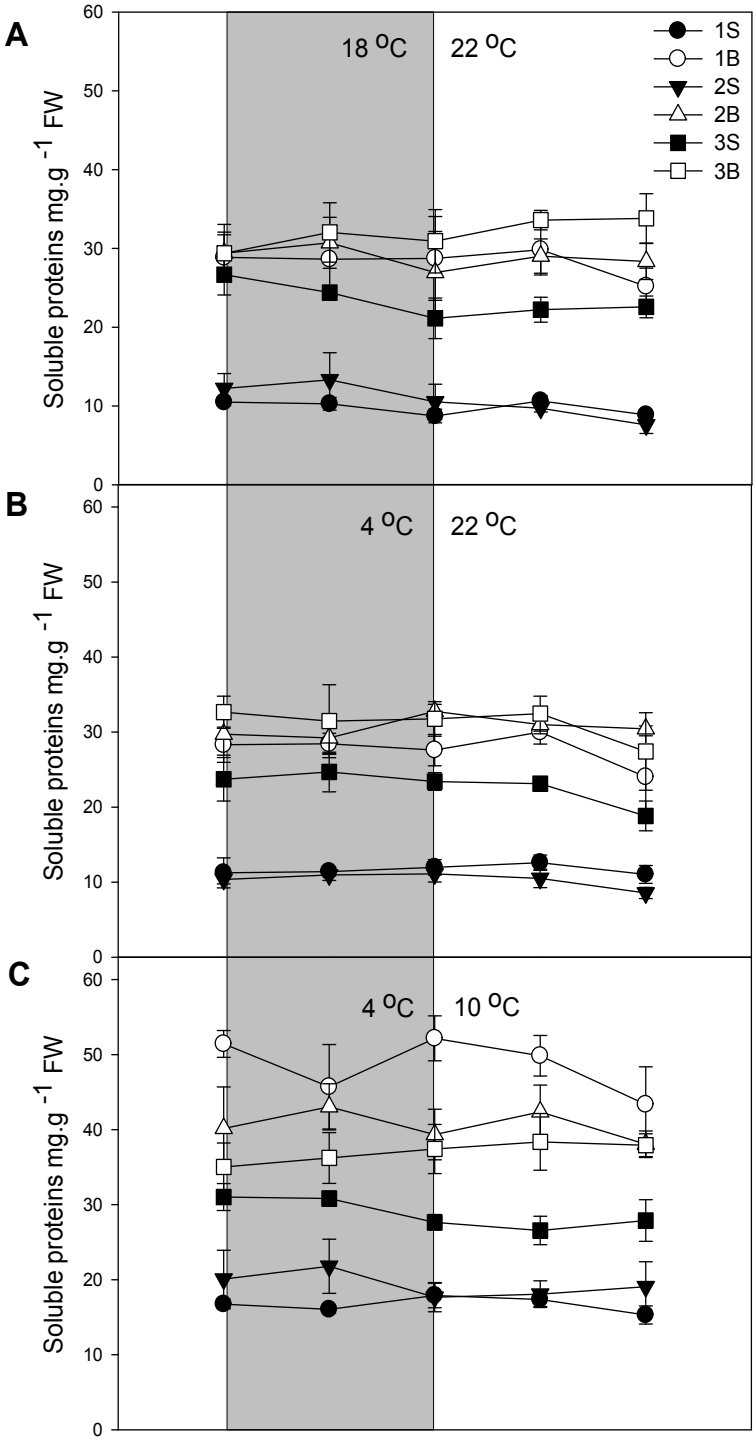


Figure 1 - Diurnal protein levels of barley grown under three temperature regimes. Protein levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

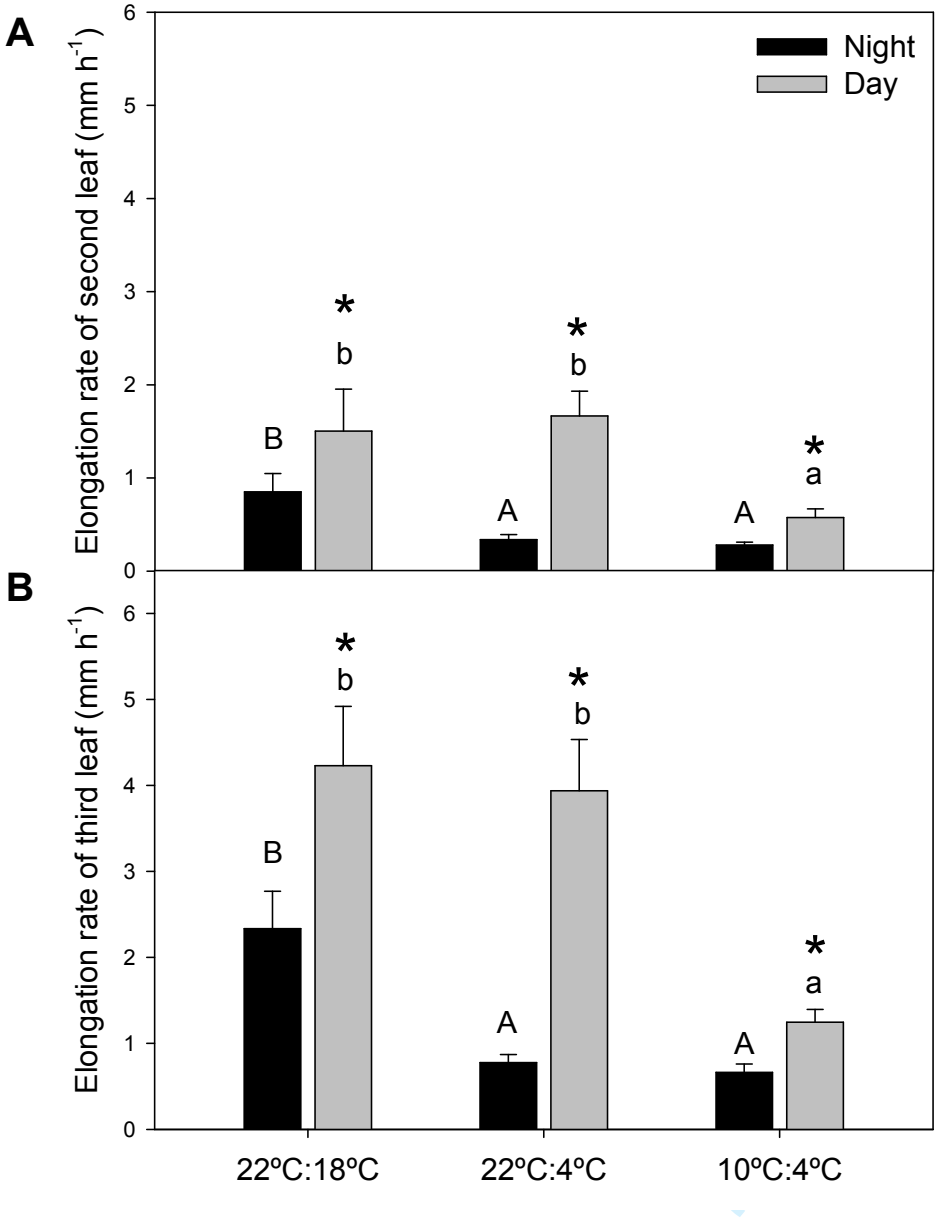


Figure 2 - Elongation rates of barley grown under three temperature regimes. (A): elongation rate of second leaves. (B): elongation rate of third leaves. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C day:night harvested at 15 DAS, 22°C:4°C day:night at 20 DAS and 10°C:4°C day:night at 42 DAS; *: difference between day and night by t-test at $P<0.05$; lowercase letter: differences between daytime measurements; uppercase letters: differences between night-time measurements; significantly different by Tukey test at $P<0.05$, error bar represents SD; n=6

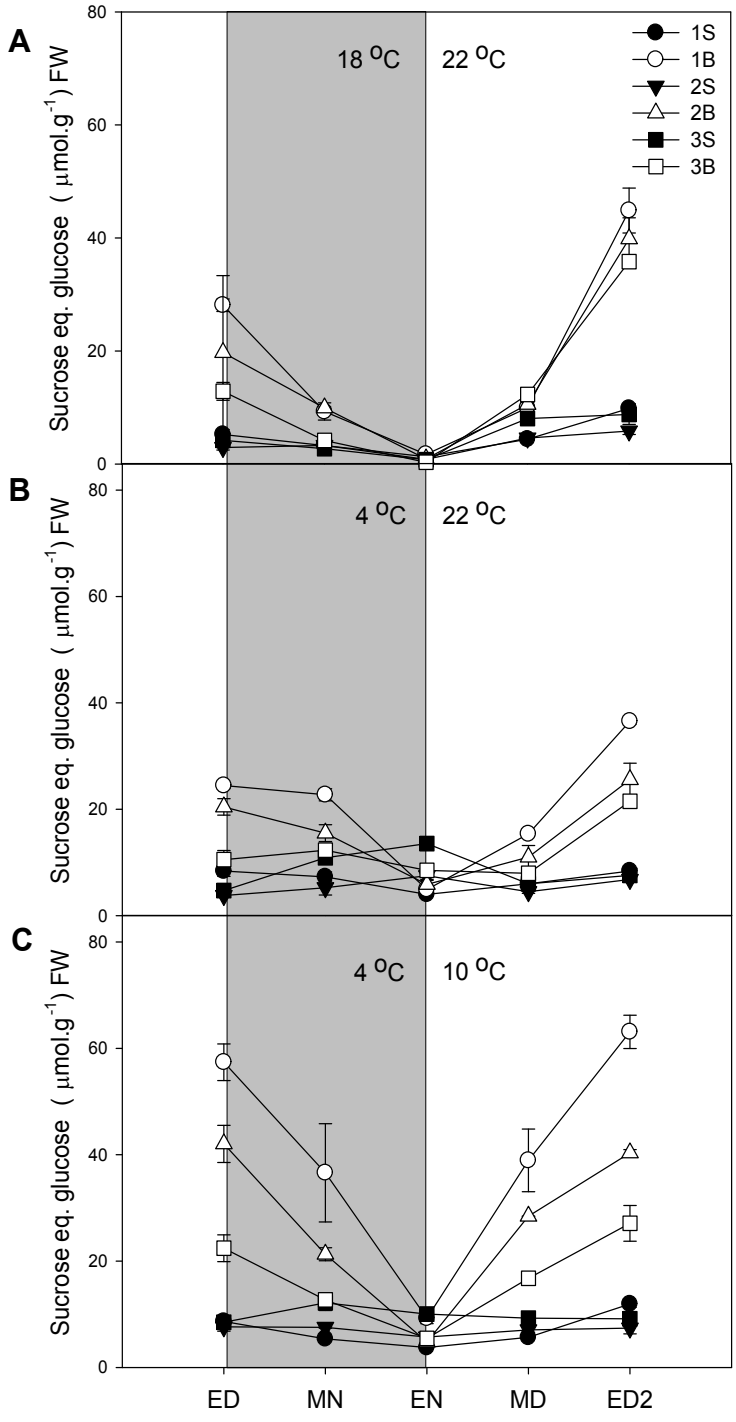


Figure 3 - Diurnal sucrose levels of barley grown under three temperature regimes. Sucrose levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

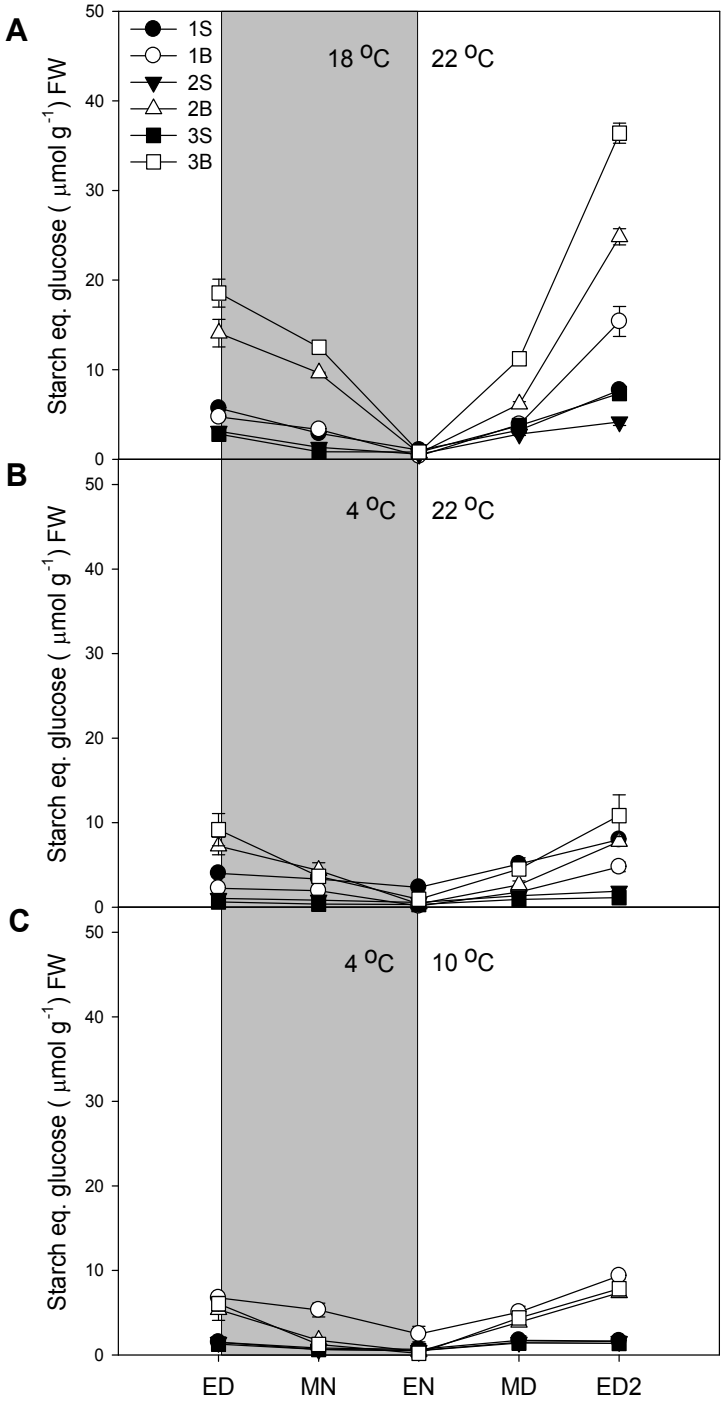


Figure 4 - Diurnal starch levels of barley grown under three temperature regimes. Starch levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

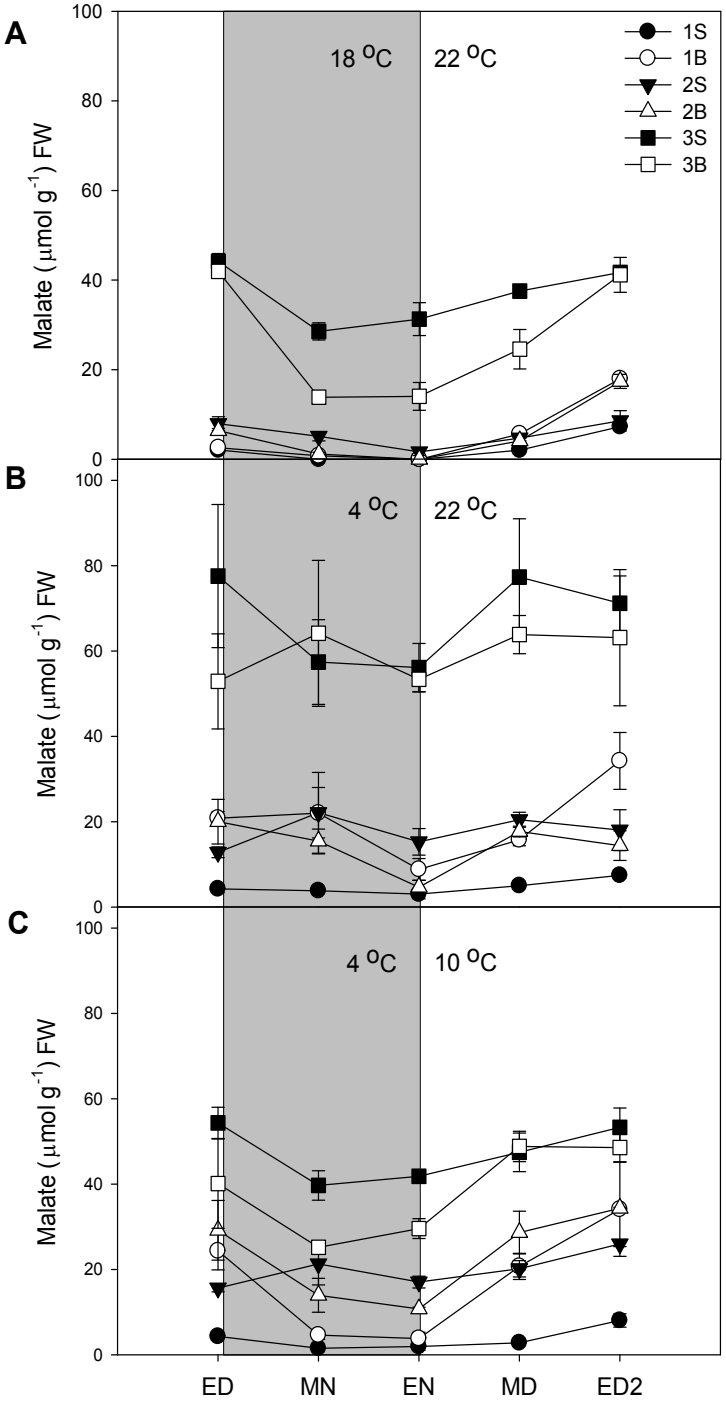


Figure 5 - Diurnal malate levels of barley grown under three temperature regimes. Malate levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

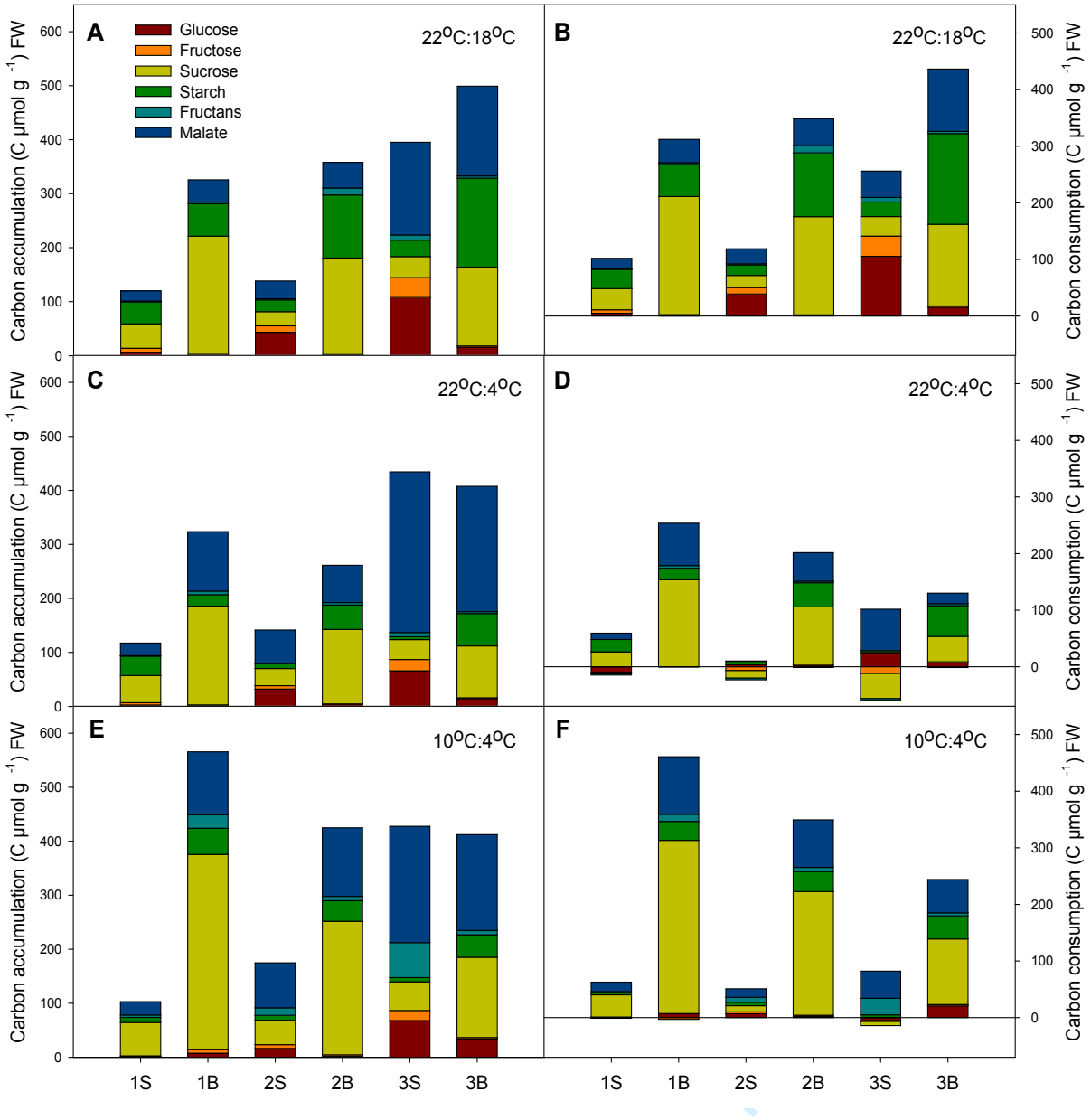


Figure 6- Carbon accumulation at end of day and carbon consumption at night per organ under three temperature regimes. Composition of carbon accumulation (A) and consumption (B) at end of the day of sheaths and blades of barley grown under 22°C:18°C day:night at 15 DAS; (C) and (D) under 22°C:4°C day:night at 20 DAS, and (E) and (F) under 10°C:4°C day:night at 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$, 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; DW: dry weight; n= 3.

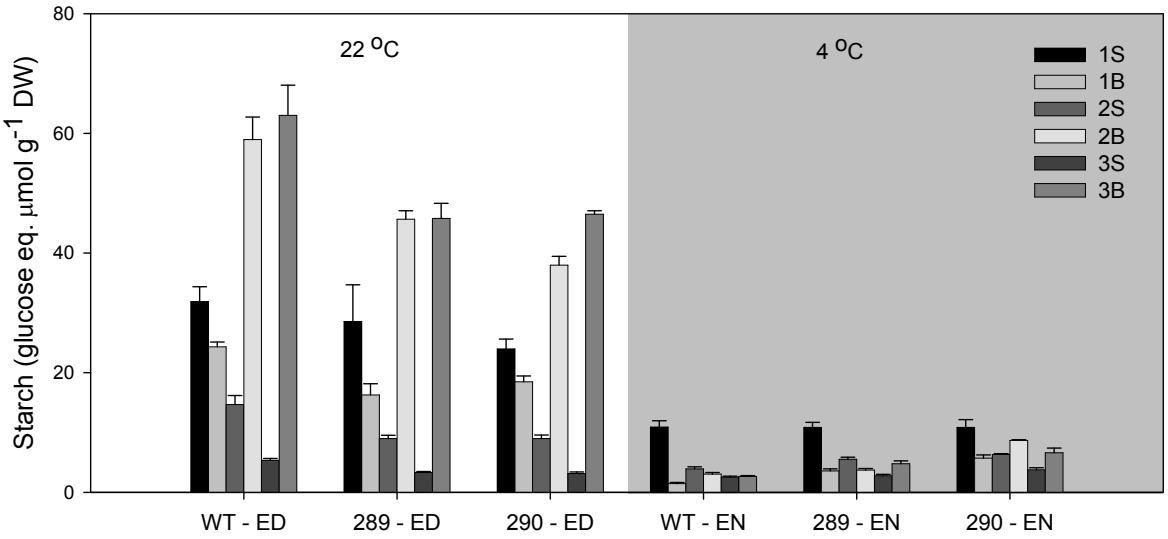


Figure 7- Diurnal starch levels of barley WT and *elf3* mutants grown under cold nights. Plants were grown under 22°C:4°C day:night, 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ for 20 DAS, until third leaf stage. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; EN: end of night; DW: fresh weight; grey panel: night period; error bar represents SD; n= 3

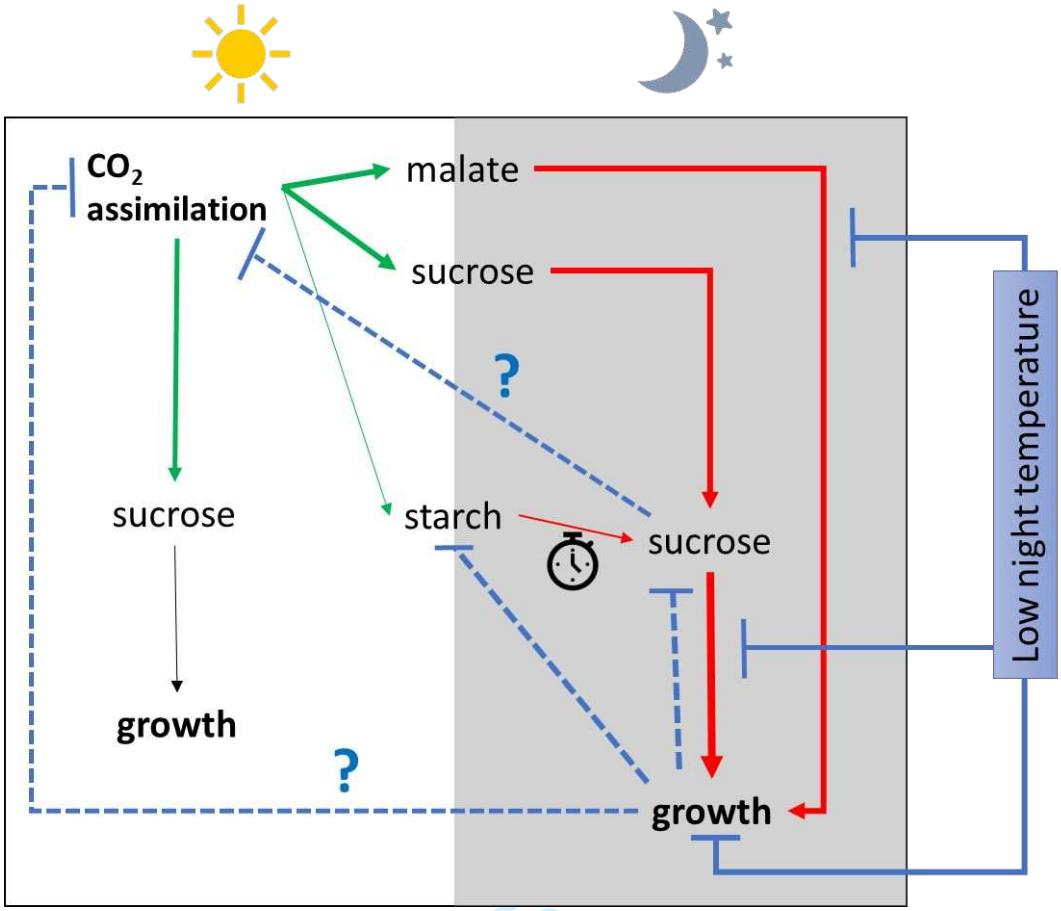
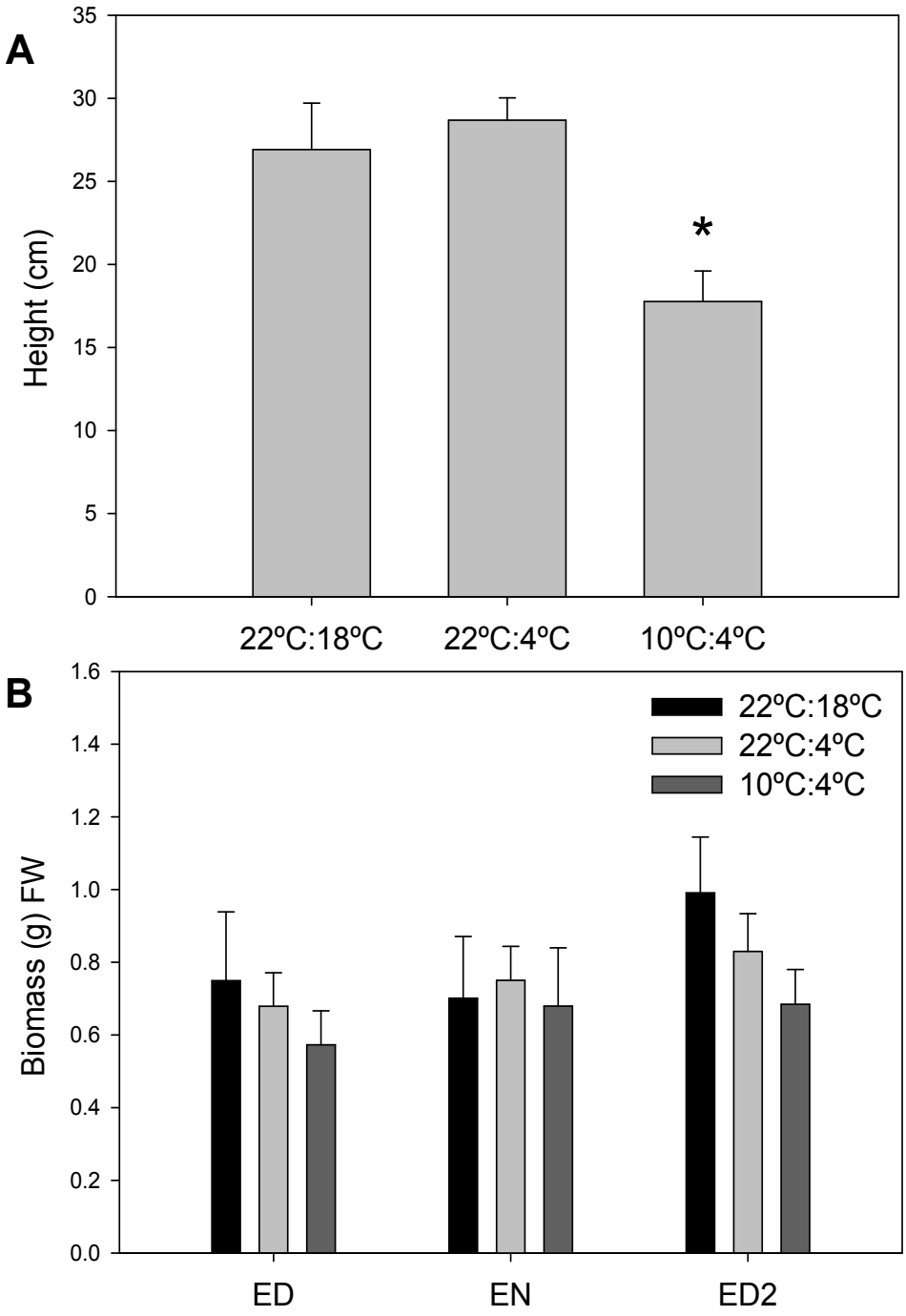


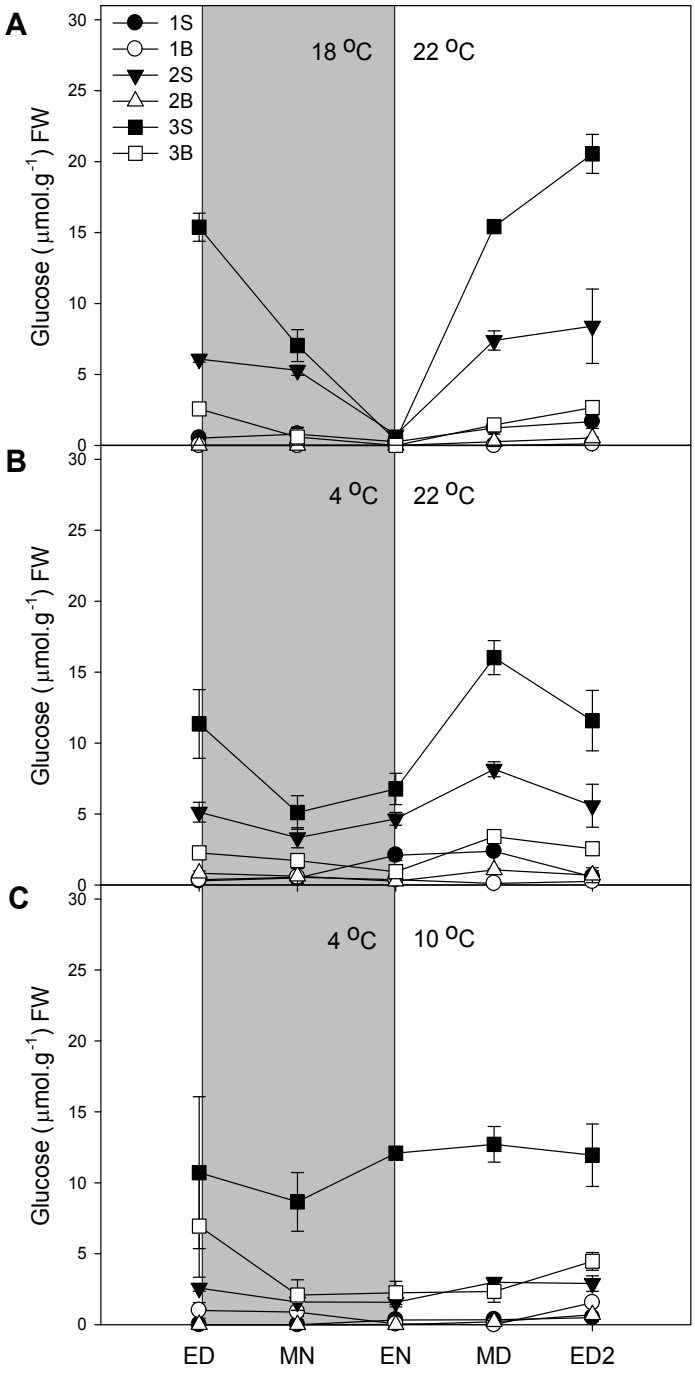
Figure 8– Schematic representation of changes in the metabolism of spring barley caused by low night temperature. During the day a large fraction of the photosynthates are accumulated in the form of sucrose and incorporated in growth. A fraction is as well accumulated, mostly as sucrose, malate and starch. Under warm nights, the growth is fuelled by sucrose, malate and starch. Sucrose and starch are almost fully consumed while malate remains with high basal levels. Under low temperature at night the growth is inhibited. It can be due to an inhibition of the mobilisation of both sucrose and especially malate stores, leading to a lack of C blocks to fuel the growth. A second hypothesis is that cold nights affects directly the growth machinery which then lead to the decrease in the mobilisation of C stores. Low night temperature also affects starch accumulation during warm days, but starch degradation at night is cold compensated, partly via a clock regulation (*elf3*).
Metabolic pathways are represented by solid lines; green: synthesis; red: degradation, thickness of lines represents proportional accumulation or degradation; blue lines: possible effects of low night temperature.

Barley uses almost equally sucrose, starch and malate for night growth under optimal growth temperatures. Under cold, mostly sucrose and malate are used, starch synthesis being strongly repressed, even when cold is only applied at night.

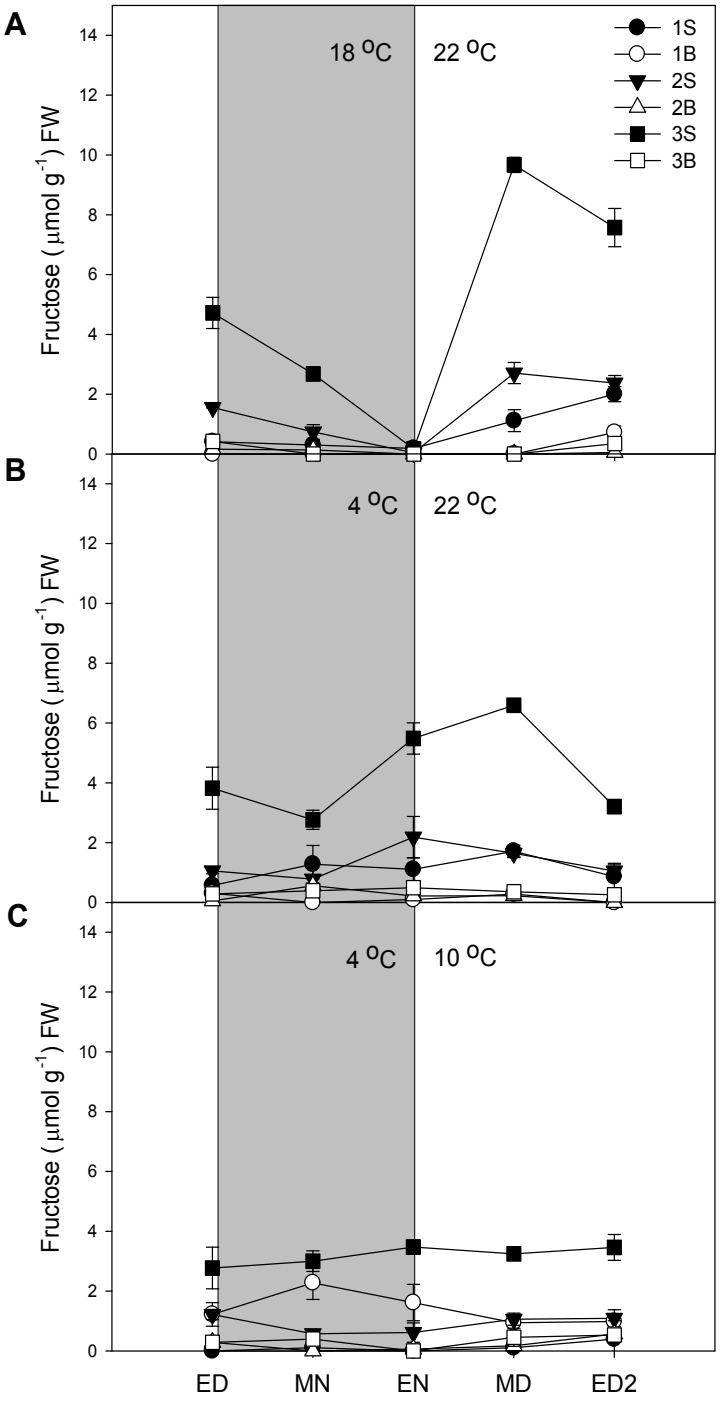


Supplemental figure S1 - Height and biomass of barley grown under three temperature regimes. (A): height at the last timepoint harvested: 22°C:18°C day:night at 15 DAS, 22°C:4°C day:night at 20 DAS and 10°C:4°C day:night at 42 DAS; (B): fresh weight of shoot biomass. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. DAS: days after sowing; error bar represents SD; n=6; ED: end of day; EN: end of night; ED2: end

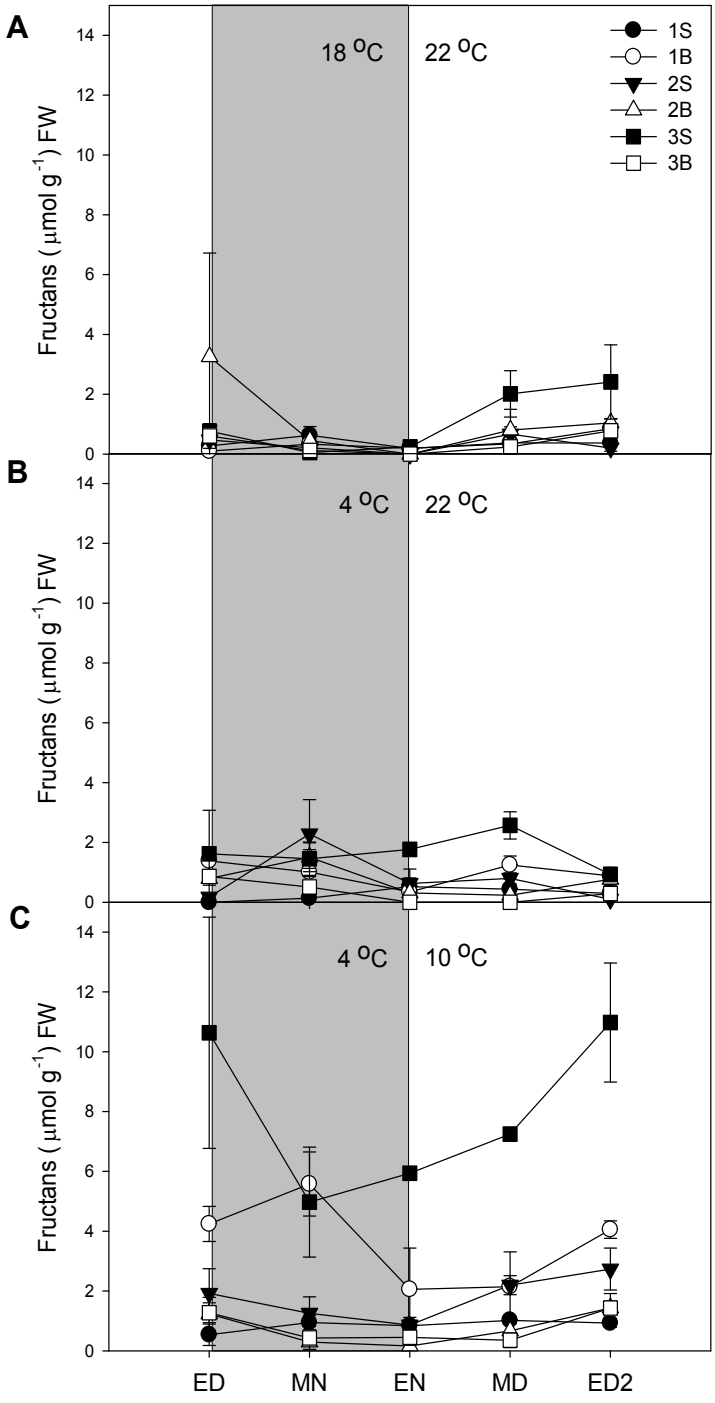
of subsequent day * represents significant difference for Tukey test at P<0.05



Supplemental figure S2- Diurnal glucose levels of plants grown under three temperature regimes. Glucose levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.



Supplemental figure S3 Diurnal fructose levels of plants grown under three temperature regimes. Fructose levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.



Supplemental figure S4- Diurnal fructan levels of plants grown under three temperature regimes. Fructan levels of plants grown under (A) 22°C:18°C day:night for 15 DAS, (B) 22°C:4°C day:night for 20 DAS and (C) 10°C:4°C day:night for 42 DAS, until third leaf stage. Plants were grown in a 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$. 1S: 1st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf sheath; 3B: 3rd leaf blade; ED: end of day; MN: middle of night; EN: end of night; MD: middle of day; ED2: end of subsequent day; FW: fresh weight; grey panels: night period; error bar represents SD; n= 3.

WARM DAY AND NIGHT 22ºC:18ºC

GLUCOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.52 ± 0.0	ABa	0.00 ± 0.0	Aa	6.09 ± 0.2	Bb	0.00 ± 0.0	Ac	15.38 ± 1.0	Ca	2.57 ± 0.3	Cd
MN	0.79 ± 0.5	ABa	0.00 ± 0.0	Aa	5.29 ± 0.3	Bb	0.00 ± 0.0	Aa	7.04 ± 1.1	Bc	0.59 ± 0.3	Aa
EN	0.26 ± 0.1	Ab	0.00 ± 0.0	Aa	0.74 ± 0.1	Ac	0.00 ± 0.0	Aa	0.37 ± 0.1	Ab	0.00 ± 0.0	Aa
MD	1.23 ± 0.4	BCbc	0.00 ± 0.0	Aa	7.39 ± 0.7	Bd	0.26 ± 0.1	Bab	15.42 ± 0.2	Ce	1.44 ± 0.3	Bc
ED2	1.67 ± 0.5	Ca	0.11 ± 0.1	Aa	8.40 ± 2.6	Bb	0.52 ± 0.1	Ca	20.55 ± 1.4	Dc	2.67 ± 0.3	Ca

FRUCTOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.41 ± 0.1	Aa	0.00 ± 0.0	Aa	1.56 ± 0.0	Cb	0.16 ± 0.0	Aa	4.72 ± 0.5	Cc	0.42 ± 0.0	Ba
MN	0.30 ± 0.2	Aab	0.00 ± 0.0	Aa	0.74 ± 0.2	Bb	0.14 ± 0.2	Aa	2.68 ± 0.2	Bc	0.00 ± 0.0	Aa
EN	0.19 ± 0.1	Aa	0.00 ± 0.0	Aa	0.04 ± 0.1	Aa	0.00 ± 0.0	Aa	0.17 ± 0.2	Aa	0.00 ± 0.0	Aa
MD	1.12 ± 0.4	Bb	0.00 ± 0.0	Aa	2.71 ± 0.4	Dc	0.00 ± 0.0	Aa	9.67 ± 0.3	Dd	0.00 ± 0.0	Aa
ED2	2.00 ± 0.3	Cb	0.72 ± 0.2	Ba	2.37 ± 0.3	Db	0.05 ± 0.1	Aa	7.57 ± 0.6	Ec	0.35 ± 0.1	Ba

SUCROSE (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	5.23 ± 0.3	Cab	28.11 ± 1.1	Cc	2.92 ± 0.5	Ba	19.73 ± 13.6	Bbc	4.18 ± 0.7	Ba	12.86 ± 1.6	Cabc
MN	3.27 ± 0.0	Ba	9.30 ± 1.5	Bb	3.28 ± 0.6	Ba	9.93 ± 0.2	ABb	2.72 ± 0.0	ABa	4.17 ± 0.1	Ba
EN	1.30 ± 0.2	Ac	1.70 ± 0.1	Ad	0.82 ± 0.2	Ab	0.89 ± 0.1	Ab	0.75 ± 0.1	Ab	0.30 ± 0.1	Aa
MD	4.40 ± 0.2	BCa	9.95 ± 0.5	Bc	4.59 ± 0.3	Cb	10.55 ± 0.3	ABc	8.04 ± 0.7	Cb	12.30 ± 0.4	Cd
ED2	9.83 ± 0.8	Da	44.86 ± 4.0	Dc	5.83 ± 0.6	Da	39.85 ± 3.7	Cbc	8.78 ± 1.8	Ca	35.80 ± 1.0	Db

STARCH (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	5.70 ± 0.2	Cb	4.73 ± 0.3	Bab	3.12 ± 0.5	Bab	14.07 ± 1.5	Dc	2.81 ± 0.3	Ba	18.55 ± 1.6	Cd
MN	2.90 ± 0.2	Bb	3.32 ± 0.3	Bb	1.33 ± 0.1	Aa	9.63 ± 0.1	Cc	0.84 ± 0.0	Aa	12.50 ± 0.3	Bd
EN	0.38 ± 0.1	Ac	0.38 ± 0.1	Aa	0.58 ± 0.1	Aab	0.68 ± 0.2	Ab	0.80 ± 0.1	Abc	0.80 ± 0.0	Cbc
MD	3.25 ± 0.2	Bab	3.89 ± 0.5	Bb	2.81 ± 0.1	Ba	6.16 ± 0.3	Bc	3.76 ± 0.3	Bb	11.21 ± 0.4	Bd
ED2	7.71 ± 0.3	Cb	15.38 ± 1.7	Cc	4.17 ± 0.4	Ca	24.83 ± 0.9	Ed	7.35 ± 0.7	Cb	36.39 ± 1.1	De

FRUCTANS (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.27 ± 0.2	Aa	0.10 ± 0.2	Aa	0.47 ± 0.1	BCa	3.25 ± 3.5	Aa	0.76 ± 0.1	ABCa	0.61 ± 0.4	BCa
MN	0.63 ± 0.3	Ab	0.33 ± 0.1	Aab	0.21 ± 0.2	ABab	0.45 ± 0.1	Aab	0.05 ± 0.1	Aa	0.12 ± 0.1	ABa
EN	0.19 ± 0.2	Aa	0.20 ± 0.2	Aa	0.00 ± 0.0	Aa	0.00 ± 0.0	Aa	0.24 ± 0.2	ABa	0.00 ± 0.0	Ba
MD	0.38 ± 0.2	Aa	0.34 ± 0.3	Aa	0.67 ± 0.2	Ca	0.80 ± 0.7	Aab	2.01 ± 0.8	BCb	0.24 ± 0.1	ABCa
ED2	0.37 ± 0.1	Aa	0.85 ± 0.0	Ba	0.20 ± 0.1	ABa	1.04 ± 0.1	Aab	2.41 ± 1.2	Cb	0.77 ± 0.2	Ca

MALATE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	2.08 ± 0.7	Ba	2.58 ± 1.0	Ba	7.96 ± 1.5	BCb	6.40 ± 0.5	Cb	44.15 ± 1.8	Cc	41.93 ± 1.0	Cc
MN	0.00 ± 0.0	Aa	0.77 ± 0.6	Aa	5.11 ± 1.0	ABCb	1.16 ± 0.4	Ab	28.54 ± 1.9	Ad	13.85 ± 0.9	Ac
EN	0.00 ± 0.0	Aa	0.00 ± 0.0	Aa	1.64 ± 0.8	Aa	0.00 ± 0.0	Aa	31.28 ± 3.7	Ac	14.05 ± 3.1	Ab
MD	2.05 ± 0.5	Ba	5.67 ± 0.7	Ca	4.72 ± 0.6	ABa	4.09 ± 0.6	Ba	37.56 ± 1.4	Bc	24.57 ± 4.4	Bb
ED2	7.29 ± 0.5	Ca	17.95 ± 0.7	Db	8.58 ± 2.3	Ca	17.38 ± 1.6	Db	41.70 ± 1.4	BCc	41.17 ± 3.9	Cc

PROTEINS (mg g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	10.49 ± 0.4	Ca	28.87 ± 2.9	Ab	12.22 ± 1.9	Ba	29.39 ± 3.7	Ab	26.66 ± 2.6	Bb	29.38 ± 2.7	Ab
MN	10.28 ± 0.8	BCa	28.64 ± 3.9	Abc	13.33 ± 3.4	Ba	30.71 ± 3.2	Abc	24.39 ± 0.5	ABb	32.03 ± 3.8	Ac
EN	8.74 ± 0.9	Aa	28.73 ± 5.3	Abc	10.53 ± 2.2	ABa	26.93 ± 5.2	Abc	21.13 ± 2.6	Ab	30.91 ± 4.0	Ac
MD	10.65 ± 0.7	Ca	29.82 ± 3.2	Acd	9.70 ± 0.5	ABa	29.02 ± 2.2	Abc	22.22 ± 1.6	Ab	33.58 ± 1.2	Ad
ED2	8.87 ± 0.4	ABa	25.19 ± 2.3	Abc	7.60 ± 1.1	Aa	28.35 ± 2.3	Ac	22.57 ± 1.4	ABb	33.82 ± 3.1	Ad

A columns between timepoints
a lines between tissues

COLD DAY AND COLD NIGHT 22ºC:4ºC

GLUCOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.00 ± 0.0	Aa	1.00 ± 0.5	Ba	2.58 ± 0.2	ABa	0.00 ± 0.0	Aa	10.72 ± 5.4	Ab	6.94 ± 3.6	Bab
MN	0.00 ± 0.0	Aa	0.89 ± 0.3	Ba	1.60 ± 0.6	Aa	0.00 ± 0.0	Aa	8.66 ± 2.1	Ab	2.09 ± 1.1	Aa
EN	0.33 ± 0.2	ABa	0.06 ± 0.1	Aa	1.58 ± 0.3	Ab	0.00 ± 0.0	Aa	12.09 ± 0.4	Ac	2.24 ± 0.8	ABb
MD	0.35 ± 0.0	ABa	0.00 ± 0.0	Aa	2.99 ± 0.2	Bb	0.21 ± 0.1	Ba	12.71 ± 1.3	Ac	2.34 ± 0.8	ABb
ED2	0.50 ± 0.3	Ca	1.55 ± 0.2	Ba	2.90 ± 0.5	Bab	0.70 ± 0.1	Ca	11.94 ± 2.2	Ac	4.47 ± 0.6	ABb

FRUCTOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.00 ± 0.0	Aa	1.22 ± 0.4	ABb	1.22 ± 0.2	Bb	0.29 ± 0.1	Aa	2.77 ± 0.7	Ac	0.28 ± 0.1	ABa
MN	0.11 ± 0.1	Aa	2.28 ± 0.6	Bb	0.58 ± 0.1	Aa	0.00 ± 0.0	Aa	3.00 ± 0.3	Ab	0.39 ± 0.0	ABa
EN	0.00 ± 0.0	Aa	1.62 ± 0.6	ABb	0.62 ± 0.3	Aa	0.06 ± 0.1	Aa	3.47 ± 0.2	Ac	0.00 ± 0.0	Aa
MD	0.11 ± 0.1	Aa	0.94 ± 0.3	Abc	1.06 ± 0.2	ABc	0.17 ± 0.0	Aa	3.25 ± 0.1	Ad	0.46 ± 0.3	Bab
ED2	0.40 ± 0.4	Aa	0.98 ± 0.4	Aa	1.09 ± 0.1	ABa	0.56 ± 0.2	Aa	3.46 ± 0.4	Ab	0.53 ± 0.2	Ba

SUCROSE (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	8.64 ± 0.7	Ca	57.39 ± 3.4	Cd	7.64 ± 0.8	Ba	42.04 ± 3.5	Dc	8.50 ± 0.6	Aa	22.42 ± 2.5	Cb
MN	5.38 ± 0.4	Ba	36.60 ± 9.2	Bc	7.55 ± 0.2	ABa	21.29 ± 1.2	Bb	12.15 ± 1.4	Bab	12.71 ± 1.1	Bab
EN	3.75 ± 0.1	Aa	9.28 ± 1.0	Ac	5.71 ± 0.6	Ab	4.80 ± 0.7	Aab	10.05 ± 0.7	ABc	5.45 ± 0.2	Aab
MD	5.68 ± 0.3	Ba	38.94 ± 5.9	Bd	7.07 ± 0.4	ABa	28.43 ± 0.0	Cc	9.27 ± 0.5	Aa	16.76 ± 0.6	Bb
ED2	11.94 ± 0.6	Da	63.10 ± 3.1	Cd	7.39 ± 1.1	ABa	40.34 ± 0.6	Dc	9.15 ± 0.0	Aa	27.10 ± 3.4	Cb

STARCH (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	1.50 ± 0.1	Ba	6.75 ± 0.2	Bb	1.48 ± 0.2	Ba	5.36 ± 1.3	Bb	1.28 ± 0.1	Ba	6.05 ± 0.9	Cb
MN	0.81 ± 0.1	Aab	5.31 ± 0.8	Bc	0.66 ± 0.1	Aa	1.73 ± 0.1	Ab	0.65 ± 0.1	Aa	1.23 ± 0.1	Aab
EN	0.67 ± 0.0	Aa	2.47 ± 0.9	Ab	0.52 ± 0.1	Aa	0.42 ± 0.0	Aa	0.50 ± 0.1	Aa	0.19 ± 0.1	Aa
MD	1.72 ± 0.1	Ba	5.06 ± 0.7	Bc	1.50 ± 0.2	Ba	3.87 ± 0.3	Bb	1.41 ± 0.1	Ba	4.38 ± 0.5	Bbc
ED2	1.66 ± 0.1	Ba	9.35 ± 0.3	Cc	1.60 ± 0.2	Ba	7.36 ± 0.3	Cb	1.36 ± 0.1	Ba	7.84 ± 0.8	Db

FRUCTANS (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.54 ± 0.4	Aa	4.24 ± 0.6	ABa	1.92 ± 0.8	ABa	1.23 ± 0.6	Ba	10.63 ± 3.9	ABb	1.27 ± 0.3	ABa
MN	0.94 ± 0.2	Aa	5.58 ± 1.1	Bb	1.26 ± 0.6	ABa	0.29 ± 0.1	Aa	4.97 ± 1.8	Ab	0.43 ± 0.4	Aa
EN	0.84 ± 0.1	Aab	2.05 ± 1.4	Ab	0.86 ± 0.3	Aab	0.16 ± 0.1	Aa	5.94 ± 0.2	ABc	0.45 ± 0.3	Aab
MD	1.02 ± 0.1	Aab	2.14 ± 1.2	Ab	2.20 ± 0.3	ABb	0.67 ± 0.3	ABa	7.24 ± 0.2	ABc	0.35 ± 0.2	Aa
ED2	0.93 ± 0.1	Aa	4.05 ± 0.3	ABb	2.73 ± 0.7	Bab	1.43 ± 0.2	Ba	10.98 ± 2.0	Bc	1.43 ± 0.5	Ba

MALATE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	4.31 ± 0.5	Ba	24.34 ± 4.4	Bb	15.64 ± 0.9	Aab	29.17 ± 7.0	Bbc	54.35 ± 3.7	Bd	40.12 ± 10.5	ABcd
MN	1.52 ± 0.0	Aa	4.61 ± 1.0	Aa	21.30 ± 4.9	ABbc	13.95 ± 4.0	Ab	39.69 ± 3.5	Ad	25.19 ± 1.1	Ac
EN	1.95 ± 0.9	ABa	3.80 ± 1.5	Aa	17.04 ± 1.4	Ac	10.75 ± 0.4	Ab	41.83 ± 1.5	Ae	29.58 ± 2.3	ABd
MD	2.79 ± 0.6	Ba	20.72 ± 3.1	Bb	20.16 ± 1.9	ABb	28.64 ± 5.0	Bb	47.45 ± 4.5	ABc	48.82 ± 3.6	Cc
ED2	8.04 ± 1.6	Ca	34.09 ± 11.0	Bb	25.96 ± 0.6	Bb	34.33 ± 0.2	Bb	53.29 ± 4.5	Bc	48.56 ± 3.3	Cc

PROTEINS (mg g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	16.75 ± 0.6	ABa	51.43 ± 1.8	ABd	20.09 ± 3.8	Aa	40.16 ± 5.5	Ac	31.02 ± 1.8	Bb	35.03 ± 3.2	Abc
MN	16.05 ± 0.5	ABa	45.72 ± 5.6	ABd	21.79 ± 3.6	Aa	43.04 ± 3.1	Acd	30.82 ± 1.0	Bb	36.22 ± 3.4	Abc
EN	17.90 ± 1.6	Ba	52.17 ± 3.0	Bd	17.67 ± 1.9	Aa	39.35 ± 3.4	Ac	27.65 ± 1.0	ABb	37.43 ± 3.3	Ac
MD	17.36 ± 0.9	ABa	49.86 ± 2.7	ABd	18.07 ± 1.8	Aa	42.37 ± 3.6	Ac	26.56 ± 1.9	Ab	38.37 ± 3.8	Ac
ED2	15.31 ± 1.2	Aa	43.37 ± 5.0	Ac	19.08 ± 3.3	Aa	38.05 ± 1.8	Ac	27.89 ± 2.8	ABb	37.93 ± 1.5	Ac

A	columns	between timepoints
a	lines	between tissues

WARM DAY AND COLD NIGHT 10ºC:4ºC

GLUCOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.30 ± 0.4	Aa	0.38 ± 0.1	Aa	5.13 ± 0.7	Ab	0.83 ± 0.2	Aa	11.35 ± 2.4	Cc	2.26 ± 0.1	Ba
MN	0.49 ± 0.3	ABa	0.55 ± 0.2	Aa	3.33 ± 0.7	Abc	0.62 ± 0.5	Aa	5.11 ± 1.2	Ac	1.72 ± 0.4	ABab
EN	2.10 ± 0.4	BCb	0.37 ± 0.3	Aa	4.66 ± 0.4	Ac	0.27 ± 0.1	Aa	6.77 ± 1.1	ABd	0.94 ± 0.3	Aab
MD	2.38 ± 1.2	Cbc	0.11 ± 0.1	Aa	8.16 ± 0.5	Bd	1.06 ± 0.1	Aab	16.02 ± 1.2	De	3.41 ± 0.3	Cc
ED2	0.57 ± 0.4	ABab	0.25 ± 0.1	Aa	5.59 ± 1.5	Ac	0.69 ± 0.5	Aab	10.61 ± 1.0	BCd	2.55 ± 0.4	BCb

FRUCTOSE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.57 ± 0.0	Aab	0.31 ± 0.1	Bab	1.06 ± 0.1	Ab	0.05 ± 0.1	Aa	3.82 ± 0.7	Ac	0.28 ± 0.1	Aab
MN	1.28 ± 0.6	ABb	0.00 ± 0.0	Aa	0.78 ± 0.5	Aab	0.56 ± 0.2	Bab	2.76 ± 0.3	Ac	0.39 ± 0.0	Aab
EN	1.11 ± 0.4	ABa	0.10 ± 0.1	Aa	2.19 ± 0.7	Bb	0.22 ± 0.1	ABa	5.48 ± 0.5	Bc	0.49 ± 0.1	Ab
MD	1.71 ± 0.2	Bb	0.28 ± 0.1	Ba	1.65 ± 0.1	ABb	0.23 ± 0.1	ABa	6.60 ± 0.1	Bc	0.36 ± 0.2	Aa
ED2	0.87 ± 0.4	ABbc	0.00 ± 0.0	Aa	1.05 ± 0.2	Ac	0.00 ± 0.0	Aa	3.20 ± 0.2	Ad	0.26 ± 0.2	Aab

SUCROSE (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	8.38 ± 1.0	Cb	24.47 ± 0.6	Cd	3.79 ± 0.3	Aa	20.44 ± 1.6	CDc	4.71 ± 0.4	Aa	10.52 ± 1.7	ABb
MN	7.29 ± 0.9	BCa	22.73 ± 1.1	Cd	5.24 ± 1.4	ABa	15.51 ± 1.6	BCc	10.87 ± 0.1	Cb	12.30 ± 1.1	Bb
EN	4.00 ± 0.4	Aa	4.92 ± 0.4	Aab	7.50 ± 0.7	Ccd	5.86 ± 0.7	Abc	13.55 ± 1.0	De	8.52 ± 0.4	Ad
MD	5.98 ± 0.2	Bab	15.38 ± 0.4	Bd	4.51 ± 0.3	Aa	11.00 ± 2.2	ABc	5.98 ± 0.6	ABab	7.96 ± 0.8	Ab
ED2	8.38 ± 0.3	Ca	36.55 ± 0.6	Dd	6.80 ± 0.2	BCa	25.57 ± 3.1	Dc	7.57 ± 0.8	Ba	21.48 ± 0.5	Cb

STARCH (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	4.00 ± 0.4	Bb	2.24 ± 0.2	Bab	1.05 ± 0.1	Ba	7.24 ± 1.0	Cc	0.62 ± 0.1	Ba	9.17 ± 1.9	Bc
MN	3.32 ± 0.1	ABc	1.95 ± 0.4	Bb	0.82 ± 0.0	ABab	4.34 ± 0.9	Bc	0.37 ± 0.0	ABa	3.64 ± 0.5	Ac
EN	2.34 ± 0.1	Ac	0.14 ± 0.0	Aa	0.56 ± 0.2	Aab	0.38 ± 0.1	Aa	0.32 ± 0.0	Aa	0.93 ± 0.3	Ab
MD	5.11 ± 0.7	Cc	1.79 ± 0.2	Bab	1.36 ± 0.1	Ca	2.63 ± 0.5	Bb	0.93 ± 0.1	Ca	4.51 ± 0.5	Ac
ED2	8.00 ± 0.3	Dcd	4.76 ± 0.6	Cb	1.89 ± 0.1	Dab	7.77 ± 0.5	Cc	1.13 ± 0.2	Ca	10.82 ± 2.5	Bd

FRUCTANS (glucose eq. µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	0.00 ± 0.0	Aa	1.38 ± 0.4	Aa	0.17 ± 0.1	Aa	0.80 ± 0.2	ABa	1.62 ± 1.5	Aa	0.86 ± 0.0	Ca
MN	0.14 ± 0.2	Aa	1.01 ± 0.7	Aab	2.28 ± 1.2	Bb	1.50 ± 0.5	Bab	1.46 ± 0.6	Aab	0.51 ± 0.3	BCab
EN	0.52 ± 0.3	Aa	0.34 ± 0.1	Aa	0.63 ± 0.5	Aa	0.31 ± 0.0	Aa	1.77 ± 0.2	Ab	0.00 ± 0.0	Aa
MD	0.44 ± 0.3	Aab	1.25 ± 0.3	Ab	0.80 ± 0.2	ABab	0.23 ± 0.3	Aa	2.57 ± 0.5	Ac	0.00 ± 0.0	Aa
ED2	0.29 ± 0.1	Aa	0.88 ± 0.3	Ab	0.10 ± 0.0	Aa	0.77 ± 0.2	ABb	0.93 ± 0.2	Ab	0.29 ± 0.1	ABa

MALATE (µmol g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	4.24 ± 0.9	Aa	20.83 ± 1.5	ABa	12.83 ± 1.3	Aa	20.02 ± 5.2	Ba	77.58 ± 16.8	Ac	52.89 ± 11.1	Ab
MN	3.79 ± 0.3	Aa	22.00 ± 9.5	ABa	22.11 ± 5.9	Aa	15.44 ± 2.8	Ba	57.41 ± 9.9	Ab	64.15 ± 17.1	Ab
EN	3.03 ± 1.1	Aa	8.82 ± 2.5	Aab	15.27 ± 3.1	Ab	4.65 ± 1.6	Aa	56.10 ± 5.7	Ac	53.36 ± 2.9	Ac
MD	4.99 ± 0.6	Aa	15.74 ± 1.4	Aa	20.45 ± 1.8	Aa	17.70 ± 1.3	Ba	77.34 ± 13.7	Ab	63.86 ± 4.5	Ab
ED2	7.43 ± 0.7	Ba	34.25 ± 6.7	Bb	18.03 ± 4.8	Aab	14.40 ± 3.5	Bab	71.21 ± 6.4	Ac	63.13 ± 16.0	Ac

PROTEINS (mg g-1 FW)

	1S		1B		2S		2B		3S		3B	
ED	11.24 ± 2.0	Aa	28.31 ± 2.3	Abc	10.36 ± 0.6	ABa	29.73 ± 2.8	Ac	23.71 ± 2.9	Bb	32.65 ± 2.1	Ac
MN	11.41 ± 0.6	Aa	28.45 ± 1.4	Abc	10.97 ± 0.7	Ba	29.23 ± 2.1	Abc	24.69 ± 2.6	Bb	31.46 ± 4.9	Ac
EN	11.98 ± 1.0	Aa	27.60 ± 2.1	Abc	11.10 ± 1.1	Ba	32.76 ± 0.9	Ad	23.41 ± 1.2	Bb	31.76 ± 2.3	Ad
MD	12.60 ± 1.0	Aa	30.00 ± 1.6	Ac	10.50 ± 1.2	ABa	31.00 ± 1.2	Ac	23.13 ± 0.4	ABb	32.45 ± 2.3	Ac
ED2	11.03 ± 1.2	Aa	24.04 ± 5.5	Abc	8.57 ± 0.7	Aa	30.44 ± 0.4	Ac	18.83 ± 2.0	Ab	27.42 ± 5.2	Ad

A	columns	between timepoints
a	lines	between tissues

Supplemental table S2- Levels of transient C reserves in shoots of cv. Bowman and *elf3* mutants day:night for 20 DAS, until third leaf stage. Values represent mean and SD. DW: dry weight; 1S: 1 sheath; 3B: 3rd leaf blade; WT: Bowman background; 289 and 290: introgression lines of *elf3*; ED: within a timepoint; small case letters represent differences between tissues within a genotype by Tu $P<0.05$, n=6.

									GLUCOSE (µ
	1S			**1B**			**2S**		
WT ED	21.25	±	4.2 Aab*	2.08	±	1 Aa	86.91	±	8.4 Bc
289 ED	16.94	±	1.7 Ab	5.05	±	0.6 Ba	31.66	±	6.8 Ac
290 ED	29.61	±	9 Ab	6.08	±	1.5 Ba	30.72	±	8.5 Ab
WT EN	11.42	±	3.5 Aa	5.6	±	2.7 Aa	80.98	±	11 ABc
289 EN	14.85	±	1.1 Aab	3.79	±	2.8 Aa	58.91	±	13.2 Ac*
290 EN	26.12	±	6.4 Bb	6.86	±	0.7 Aa	105.32	±	9.3 Bc*
									FRUCTOSE (
	1S			**1B**			**2S**		
WT ED	15.12	±	2.3 Babc*	3.38	±	1.6 Aa	21.68	±	1.7 Bbc
289 ED	9.27	±	2.5 Ab	2.07	±	1.8 Aa	11.65	±	2.3 Ab
290 ED	11.99	±	2.1 ABb	1.67	±	2.5 Aa	12.62	±	3.7 Ab
WT EN	5.64	±	2.8 Aa	5.48	±	1 ABa	46.26	±	5.7 ABb*
289 EN	11.09	±	0.4 ABa	3.93	±	1.2 Aa	30.61	±	2.1 Ab*
290 EN	17.72	±	5.3 Ba	7.87	±	1 Ba*	59.35	±	9.5 Bb*
									SUCROSE (eq. glu
	1S			**1B**			**2S**		
WT ED	39.94	±	5.8 Ab*	262.24	±	26.9 Ad*	22.01	±	6.3 Aab
289 ED	49.53	±	7.2 Ab	229.43	±	8.2 Ad*	34.97	±	4.9 Ab
290 ED	52.07	±	1.4 Ab*	237.43	±	7.6 Ad*	31.04	±	11.5 Aa
WT EN	21.57	±	1.3 Ab	33.49	±	0.5 Ac	61.43	±	5.5 Bd*
289 EN	41.66	±	7.3 Bb	52.86	±	7.8 Bbc	66.21	±	6 Bc*
290 EN	31.93	±	7.8 ABb	87.15	±	5.4 Cd	44.94	±	5.8 Abc
									STARCH (eq. gluc
	1S			**1B**			**2S**		
WT ED	31.86	±	2.5 Ac*	24.32	±	0.8 Bc*	14.67	±	1.5 Bb*
289 ED	28.56	±	6.1 Ac*	16.28	±	1.9 Ab*	8.99	±	0.5 Aab*
290 ED	23.99	±	1.6 Ad*	18.48	±	1 Ac*	8.99	±	0.6 Ab*
WT EN	10.92	±	1 Ad	1.46	±	0.2 Aa	3.93	±	0.3 Ac
289 EN	10.86	±	0.8 Ad	3.58	±	0.3 Bab	5.53	±	0.3 Bc
290 EN	10.85	±	1.3 Ad	5.72	±	0.5 Cb	6.32	±	0.1 Cb
									FRUCTANS (eq. glu
	1S			**1B**			**2S**		
WT ED	5.29	±	2.3 Ba	37.17	±	4 Bb*	10.78	±	4.3 Ba
289 ED	0	±	0 Aa	25.06	±	1.8 Ae*	0.45	±	0.8 Aab
290 ED	4.13	±	3 Ba	34.38	±	6.1 ABc*	6.64	±	1.7 ABa
WT EN	3.71	±	0.6 Aa	6.35	±	1.1 Aab	12.09	±	0.8 Bb
289 EN	0.58	±	1 Aa	13.01	±	2.7 Aab	8.44	±	2.4 ABa*
290 EN	2.46	±	2.6 Aa	9.91	±	7.6 Aa	2.98	±	3.9 Aa

									MALATE (µ
	1S			**1B**			**2S**		
WT ED	413.37	±	10 Aa*	637.64	±	32.8 Bb*	456.56	±	12.1 Aa
289 ED	449.85	±	26.4 Aa*	531.53	±	16.2 Abc	476.68	±	31.6 Aab
290 ED	526.84	±	78.7 Aa	550.36	±	25.4 Aa	459.51	±	48.4 Aa
WT EN	331.7	±	14.6 Aa	510.73	±	13.7 Ac	440.74	±	29.3 Ab
289 EN	379.83	±	23.8 Ba	551.82	±	44.4 Ab	439.83	±	57 Aa
290 EN	440.15	±	11.6 Ca	471.67	±	56.2 Aab	482.47	±	15 Aab
									PROTEINS (
	1S			**1B**			**2S**		
WT ED	76.48	±	10.6 Aa	233.28	±	1.7 Ac	97.81	±	21.7 Aa
289 ED	66.41	±	15.7 Aa	243.64	±	10.7 Acd	129.68	±	5.6 Ab
290 ED	105.06	±	26.6 Aa	238.57	±	8.4 Abc	124.54	±	14.3 Aa
WT EN	101.28	±	16.6 Aa	254.37	±	5.2 Ad*	154.88	±	16 Ab*
289 EN	98.89	±	9.2 Aa*	236.75	±	14.2 Abc	123.77	±	23 Aa
290 EN	59.19	±	57.8 Aa	233.53	±	6.7 Abc	140.69	±	42.5 Aab

under 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:4°C
st leaf sheath; 1B: 1st leaf blade; 2S: 2nd leaf sheath; 2B: 2nd leaf blade; 3S: 3rd leaf
end of day; EN: end of night. Capital letters represent differences between genotypes
ukey $P<0.05$; * represents differences between timepoints within a genotype by t-test

µmol g^{-1} DW)		
2B	**3S**	**3B**
22.76 ± 3.8 Bab	143.57 ± 16.4 Bd	39.26 ± 6.6 Ab
12.08 ± 2.6 Aab	92.33 ± 5.6 Ad	30.93 ± 0.7 ABc
13.5 ± 1.8 Aa	101.99 ± 0.2 Ac	28.22 ± 3.1 Bb
16.01 ± 4.6 Aa	141.22 ± 8.3 Bd	47.27 ± 3.2 Bb
8.99 ± 1.1 ABa	106.93 ± 7.8 Ad	29.96 ± 5.8 Ab
16.22 ± 1.4 Bab	134.47 ± 7.2 Bd*	29.65 ± 5 Ab
µmol g^{-1} DW)		
2B	**3S**	**3B**
11.5 ± 1.5 ABab	47.13 ± 1.4 Bd	28.14 ± 11.9 Ac
8.46 ± 2 Ab	39.86 ± 2.5 Ad	18.31 ± 1.5 Ac
13.42 ± 0.6 Bb	41.2 ± 1.8 Ac	18.41 ± 3.2 Ab
10.21 ± 4.2 Ab	91.97 ± 8.9 Bc*	38.35 ± 5 Bb
10.58 ± 0.3 Aa	66.8 ± 12.9 Ac*	30.8 ± 7.9 ABb
16.5 ± 1.8 Aa*	85.25 ± 4 ABc*	21 ± 1.9 Aa
cose µmol g^{-1} DW)		
2B	**3S**	**3B**
160.93 ± 3 Ac*	0 ± 0 Aa	56.53 ± 17.8 Ab*
166.61 ± 13.8 Ac*	11.07 ± 8.1 Aa*	42.68 ± 4.2 Ab*
171.38 ± 4.5 Ac*	12.68 ± 10.9 Aa*	69.4 ± 3.5 Ab*
7.32 ± 3.8 Aa	0 ± 0 Aa	3.19 ± 4.8 Aa
41.27 ± 5.8 Bb	0 ± 0 Aa	9.8 ± 6.1 Aa
55.3 ± 3.1 Cc	0 ± 0 Aa	1.18 ± 2 Aa
cose µmol g^{-1} DW)		
2B	**3S**	**3B**
58.96 ± 3.8 Cd*	5.35 ± 0.3 Ba*	63.02 ± 5 Bd*
45.65 ± 1.4 Bd*	3.32 ± 0.2 Aa*	45.8 ± 2.5 Ad*
37.98 ± 1.5 Ae*	3.15 ± 0.3 Aa	46.48 ± 0.6 Af*
3.05 ± 0.3 Abc	2.56 ± 0.1 Aab	2.67 ± 0.1 Aabc
3.71 ± 0.3 Bab	2.78 ± 0.2 Aa	4.78 ± 0.5 Bbc
8.68 ± 0.1 Cc	3.78 ± 0.3 Ba	6.62 ± 0.8 Cb
ucose µmol g^{-1} DW)		
2B	**3S**	**3B**
15.22 ± 7.6 Aa*	15.82 ± 3.6 Aa	9.8 ± 8.5 Aa
19.22 ± 1.6 Ade*	14.64 ± 6 Acd	7.67 ± 1.8 Abc
15.16 ± 6.6 Aab	19.94 ± 2.7 Ab	5.12 ± 2.1 Aa
0.88 ± 1.5 Aa	26.77 ± 4.9 Ac*	3.35 ± 0.6 Aa
2.1 ± 3.6 ABa	21.94 ± 3.5 Ab	1.84 ± 3.2 Aa
7.62 ± 1.8 Ba	16.93 ± 17 Aa	6.85 ± 4.4 Aa

μmol g^{-1} DW)								
2B			**3S**			**3B**		
588.94	± 23.9	Bb*	712.39	± 29.8	Bc	606.36	± 17.5	Bb*
499.6	± 9.5	Aab	733.27	± 44.1	Bd	601.21	± 31.6	Bc
530.26	± 17	Aa	573.56	± 43.4	Aa	475.5	± 8.3	Aa
452.02	± 27.2	Ab	808.02	± 22.6	Ad*	523.11	± 3.8	Bc
564.18	± 4.1	Bb*	729.28	± 63.6	Ac	566.8	± 4.7	Bb
546.72	± 26.4	Bb	766.25	± 20.4	Ac*	404	± 52.3	Aa
(mg g^{-1} DW)								
2B			**3S**			**3B**		
230.59	± 23.9	Ac	171.12	± 20.5	Ab	258.17	± 9	Ac
269.54	± 8.6	Ad*	213.3	± 20.7	ABc	240.68	± 25.8	Acd
259.39	± 14	Abc	222.97	± 7.2	Bb*	272.99	± 13.1	Ac
270.75	± 14.4	Ad	214.09	± 11.3	Ac*	256.76	± 4.6	Ad
235.19	± 13.9	Abc	191.36	± 26.9	Ab	264.11	± 21.5	Ac
253.3	± 14.9	Ac	169.16	± 20.9	Abc	208.57	± 42.4	Abc

Supplemental table S3 - Levels of transient C reserves in crown of cv. Bowman, *elf3* mutants and cv. Propino under 12h:12h light:dark photoperiod with 500 µmol photons $m^{-2}s^{-1}$ under 22°C:18°C, 22°C:4°C and 10°C:4°C day:night until third leaf stage. Values represent mean and SD. Glucose, fructose and malate are given in µmol g^{-1} FW, sucrose, starch and fructans are given in µmol g^{-1} FW, proteins are given in mg g^{-1} FW. ED: end of day; EN: end of night; WT: Bowman background; 289 and 290: introgression lines of *elf3*; PRO: cv. Propino; FW: fresh weight.Capital letters represent differences between genotypes within a timepoint by Tukey $P<0.05$; * represents differences between timepoints within a genotype by t-test $P<0.05$, n=6.

22 ºC:18 ºC

	GLUCOSE	FRUCTOSE	SUCROSE	STARCH	FRUCTANS	MALATE	PROTEINS
WT ED	0.70 ± 0.2 B*	0.42 ± 0.1 A	0.31 ± 0.1 A	2.49 ± 0.4 AB*	7.35 ± 1.4 A*	19.74 ± 3.8 C*	18.34 ± 2.3 A
289 ED	0.52 ± 0.2 AB*	0.29 ± 0.1 A	0.34 ± 0.1 AB*	1.89 ± 1.1 A	10.00 ± 2.7 AB*	11.30 ± 3.5 AB	19.44 ± 0.9 A
290 ED	0.25 ± 0.1 A	0.32 ± 0.2 A	0.26 ± 0.1 A	1.40 ± 0.5 A*	9.95 ± 2.1 AB*	9.05 ± 3.5 A	18.49 ± 1.8 A
PRO ED	0.69 ± 0.2 B	0.55 ± 0.2 A	0.49 ± 0.1 B*	3.37 ± 0.6 B	13.20 ± 1.1 B*	17.18 ± 2.7 BC*	20.68 ± 0.5 A*
WT EN	0.20 ± 0.0 A	0.24 ± 0.0 A	0.25 ± 0.1 AB	1.05 ± 0.4 A	4.08 ± 1.4 A	10.84 ± 2.2 AB	17.00 ± 1.9 AB
289 EN	0.20 ± 0.1 A	0.13 ± 0.0 A	0.13 ± 0.0 A	0.87 ± 0.3 A	4.20 ± 1.0 A	8.43 ± 1.5 A	18.67 ± 1.3 AB
290 EN	0.32 ± 0.2 AB	0.30 ± 0.1 AB	0.19 ± 0.0 AB	0.37 ± 0.2 A	2.23 ± 0.8 A	9.13 ± 3.0 A	15.27 ± 3.6 A
PRO EN	0.48 ± 0.2 B	0.46 ± 0.2 B	0.27 ± 0.1 B	2.78 ± 0.5 B	8.80 ± 2.1 B	13.71 ± 1.8 B	19.60 ± 0.9 B

22 ºC:4 ºC

	GLUCOSE	FRUCTOSE	SUCROSE	STARCH	FRUCTANS	MALATE	PROTEINS
WT ED	1.31 ± 0.3 A	0.67 ± 0.2 A	2.36 ± 0.3 A	1.09 ± 0.4 A	10.86 ± 2.2 C	19.96 ± 1.6 A	18.07 ± 4.2 A
289 ED	0.99 ± 0.6 A	0.55 ± 0.3 A	2.07 ± 0.5 A	0.77 ± 0.3 A	5.09 ± 1.8 A	17.83 ± 5.1 A	21.20 ± 3.0 A
290 ED	1.00 ± 0.1 A	0.55 ± 0.2 A	1.92 ± 0.4 A	0.89 ± 0.2 A	7.85 ± 1.1 B	16.61 ± 3.7 A	22.63 ± 1.7 A*
PRO ED	1.54 ± 0.3 A	0.73 ± 0.2 A	2.32 ± 0.7 A	1.95 ± 0.1 A*	10.89 ± 0.7 C	18.91 ± 5.2 A	21.45 ± 2.9 A
WT EN	2.25 ± 0.4 B*	1.09 ± 0.3 A*	4.66 ± 0.9 B*	0.77 ± 0.2 A	22.38 ± 2.9 A*	19.99 ± 2.9 A	19.72 ± 2.4 AB
289 EN	1.61 ± 0.1 A	1.07 ± 0.2 A*	4.87 ± 1.6 B*	0.61 ± 0.1 A	22.66 ± 2.9 A*	21.65 ± 1.7 A	20.59 ± 2.1 B
290 EN	1.73 ± 0.3 A	0.70 ± 0.1 A	2.45 ± 0.3 A	0.68 ± 0.2 A	20.73 ± 1.6 A*	21.75 ± 3.2 A	16.51 ± 1.4 A
PRO EN	2.09 ± 0.2 AB	1.03 ± 0.2 A*	3.17 ± 1.5 AB	1.17 ± 0.1 A	19.67 ± 0.9 A*	24.02 ± 4.9 A	20.71 ± 1.0 B

10 ºC:4 ºC

	GLUCOSE	FRUCTOSE	SUCROSE	STARCH	FRUCTANS	MALATE	PROTEINS
WT ED	1.48 ± 0.2 A	1.23 ± 0.1 A	16.38 ± 1.2 A	3.48 ± 0.8 A	16.88 ± 3.1 A	17.70 ± 1.6 AB	24.08 ± 1.8 A
289 ED	1.11 ± 0.1 A	1.09 ± 0.1 A	16.82 ± 2.1 A	4.04 ± 0.6 A	29.22 ± 2.5 A*	16.81 ± 0.7 A	23.79 ± 2.0 A*
290 ED	1.39 ± 0.3 A	1.29 ± 0.2 A	16.99 ± 1.6 A	3.57 ± 0.6 A	18.85 ± 4.6 A	12.23 ± 1.7 A	22.94 ± 1.3 A
PRO ED	2.00 ± 0.4 B	1.57 ± 0.1 B	19.09 ± 1.1 A	6.40 ± 0.7 A	24.87 ± 1.9 A	23.45 ± 1.6 B*	25.83 ± 2.9 A
WT EN	1.38 ± 0.2 A	1.17 ± 0.1 A	24.40 ± 0.7 A*	2.88 ± 0.6 A	17.79 ± 3.0 A	18.59 ± 0.6 A	21.61 ± 1.9 A
289 EN	1.38 ± 0.2 A*	1.40 ± 0.1 AB*	24.55 ± 2.3 A*	4.02 ± 0.5 AB	18.00 ± 2.5 A	18.24 ± 0.5 A*	21.05 ± 2.2 A
290 EN	2.16 ± 0.4 B*	1.71 ± 0.1 B*	26.85 ± 3.4 A*	3.32 ± 0.6 AB	27.33 ± 7.9 A	15.02 ± 1.9 B*	21.36 ± 1.7 A
PRO EN	2.40 ± 0.3 B	1.74 ± 0.2 AB	24.77 ± 1.8 A*	6.27 ± 0.5 B	23.69 ± 5.1 A	21.04 ± 0.8 B	23.98 ± 2.5 A

A = comparison between genotypes within timepoint

* = comparison between timepoints within genotype